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The effect on milk yield of three times a day milking and of increasing the level of residual milk

By GILLIAN M. ELLIOTT*

National Institute for Research in Dairying, Shinfield, Reading

(*Received* 10 *November* 1960)

Summary. Experiments were made to determine the effects on yield and butterfat percentage of three times a day milking and of inefficient milking using cows whose milk secretion rates had been shown to be unaffected by the milk accumulation involved. The milking treatments were applied to half-udders of the same cow to ensure that there was no difference between each pair of experimental units in the amount of oxytocic hormone released by the pituitary gland.

Milking three times a day compared with twice a day over a 39-day period resulted in an increase of 12% in both milk and butterfat yields. Increasing the residual milk by 1 lb in each half-udder for a 39-day period caused a 15% decrease in both milk and butterfat yields. It was concluded that since the cause of these findings could be neither the direct effect of differences in milk accumulation nor the effect of different amounts of oxytocic hormone, some local cell effect was involved, activated either by differences in the amount of residual milk or by long-term effects of differences in milk accumulation.

Three times a day milking and efficient milking (of the sort that would reduce the amount of residual milk in the udder) have always been considered practices which would increase milk production. This has not in the past been seriously questioned since it has seemed reasonable on the basis that both would relieve milk accumulation in the udder. However, since the publication of evidence suggesting that the rate of milk secretion is constant during milking intervals of normal length and that relief of milk accumulation should therefore carry no benefit (Elliott, Dodd & Brumby, 1960; Elliott, 1959), some doubt has been cast on the extent of the advantages of these two milking practices and some interest expressed as to the true cause of their effect (Elliott, 1959). Two possible causes of the observed facts have been suggested, that of the difference in the amount of oxytocic hormone released by the pituitary gland, and that of the difference in the amount of residual milk retained in the udder.

In the following experiments, three times a day milking was compared with twice a day milking (Experiment 1) and delayed milking after stimulation of ejection with no delay (Experiment 2) using cows whose milk secretion rates had been shown in a preliminary experiment to be unaffected by the degree of milk accumulation involved. To distinguish between the two suggested causes of any effect the treatments

* Animal Health Trust Wellcome Research Fellow, 1956–1958.

might have on milk yield, the first—that of oxytocic hormone difference—was eliminated by the use of half-udders as experimental units, and the second—that of residual milk difference—was allowed to play its part as a difference between the treatments.

EXPERIMENTAL

Eleven Ayrshire heifers and one Shorthorn cow were used. In Experiments 1 and 2 a half-udder milking machine was used. In this way the milking of each half of the udder could proceed independently, and the milk from each half could be kept separate. The two clawpieces could be clipped together if both udder halves were being milked at the same time, but otherwise the half-udder milking machines were used quite separately. Ruakura milk flow indicators were used. All milking and stripping was done by machine in a two-level parlour. A vacuum of 15 inHg and a pulsation ratio of 3:1 were used.

Preliminary experiment to determine the period of milk accumulation during which the rate of milk secretion remained constant

The experimental design used was a duplicated six cows × six treatments balanced Latin square similar to those described by Elliott *et al.* (1960). The same randomization of treatments was used for the two squares. The treatments were milking intervals of 8, 10, 12, 14, 16 and 18 h duration, and the intervening constant length interval was 8 h after treatment intervals of 8, 10 and 12 h and two successive intervals of 8 h each after treatment intervals of 14, 16 and 18 h. All cows began the experiment with an interval of 8 h. Ten i.u. of synthetic oxytocic hormone ('Syntocinon', Sandoz Products Ltd) was given at every milking and was injected through a polythene mammary vein cannula in a way similar to that described by Elliott *et al.* (1960).

Results

The mean effect of each of the six treatment intervals on milk and butterfat secretion and on butterfat percentage is shown for both groups of six cows (nos. 1 and 2) in Fig. 1. Fig. 2 shows the effect of the treatment intervals on yield in the following one or two milking intervals of 8 h.

Discussion

The milk and butterfat secretion rates of the six cows on Latin square no. 1 and the six on Latin square no. 2 appeared to remain constant during a period of accumulation of up to 14 h, and for yields of up to 23 lb. The secretion rates of both groups appeared to be unaffected by preceding intervals of up to 14 h.

Experiment 1. *The effect on yield and butterfat percentage of increasing the frequency of daily milking from twice to three times*

The two treatments were twice a day milking at 12 h intervals and three times a day at 8 h intervals, and were applied to the same cow at the same time by using udder halves as the experimental units.

The animals used were the six Ayrshire heifers which had been on Latin square

no. 1 in the preliminary experiment. They had been in milk 110 days on average and produced on average 16 lb of milk in 12 h at the beginning of the experiment, and 12 lb in 12 h at the end. Any difference in yield between the halves was determined during a pre-experimental period of 9 days by twice daily milking. The experimental period lasted for 39 days, during which time the left half of the udder of each cow was milked three times and the right half twice a day. This was followed by a further 9 days during which both halves were again milked twice daily (post-experimental

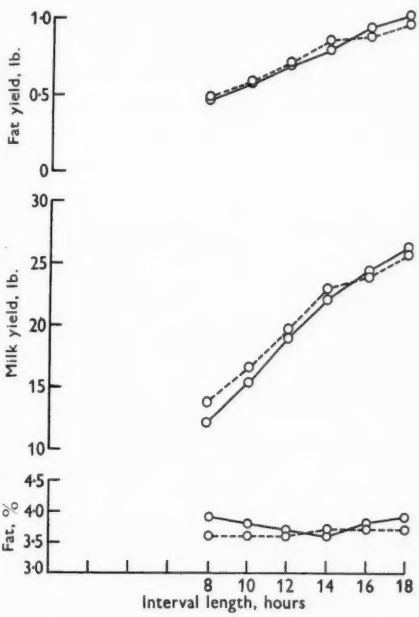

Fig. 1

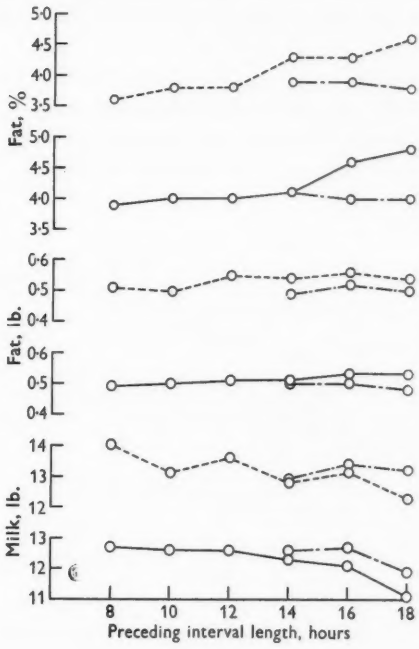

Fig. 2

Fig. 1. Milk and butterfat yields, and the butterfat content of the milk, obtained after milking intervals of 8 to 18 h. Means of six cows, preliminary experiment. O—O, Latin square no. 1; O---O, Latin square no. 2.

Fig. 2. The yields of milk and butterfat, and the butterfat content of the milk, obtained in the constant intervening intervals between the variable milking intervals. Means of six cows, preliminary experiment. O—O, Latin square no. 1, first interval of 8 h; O---O, Latin square no. 2, first interval of 8 h; O·····O, second interval of 8 h.

period). During the pre-experimental and post-experimental periods milking was done at 6 a.m. and 3 p.m. During the experimental period the cows were brought into the milking shed four times during the day, both halves milked at 6 a.m., the left half again at 2 p.m. and 10 p.m., and the right half again at 6 p.m. Immediately a cow entered the bail she began to feed. Without delay the udder was washed, a foremilk sample taken and the machine placed on the teats immediately. When one half only of the udder was being milked, only that half was washed and sampled. Stripping was begun when the milk-flow indicator showed that the rate had dropped to less than 0·5 lb/min.

The milk yield from each udder half was recorded at every milking. Three-day composite butterfat samples were taken, a quantity of milk proportional to yield being taken at every milking.

The cows were kept in covered yards where they received a maintenance ration of hay and brewer's grains. Cattle cake was fed at the rate of 12 lb per day per cow regardless of yield. This simplified rationing was used partly because the cows had very similar yields (about 32 lb/day), and partly because accurate rationing according to yield was not considered necessary in a half udder experiment. The 12 lb of cake was fed by giving 3 lb at each of the four milkings.

Results

All milk yields are presented as mean daily yields in 3-day periods. Periods 1–3 inclusive comprise the pre-experimental period, 3–16 the experimental period, and 17–19 the post-experimental period.

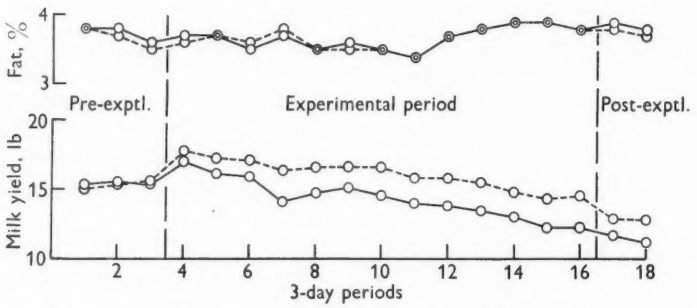

Fig. 3. Mean daily milk yield and butterfat percentage of udder halves in 3-day periods. Means of six cows, Experiment 1. O—O, Twice a day milking; O···O, three times a day milking.

Table 1. *Mean daily milk yield and butterfat percentage of the udder halves*

	Left half (3×)	Right half (2×)	Difference in favour of 3×	Corrected difference	% corrected difference
Milk yield, lb					
Pre-experimental period	15·3	15·5	−0·2	0	—
Experimental period	16·0	14·4	1·6	1·8	12
Post-experimental period*	12·9	11·5	1·4	1·6	—
Butterfat, %					
Pre-experimental period	3·7	3·7	0	—	—
Experimental period	3·7	3·7	0	—	—
Post-experimental period*	3·8	3·9	−0·1	—	—

* The mean results in the post-experimental period consist of two 3-day periods only because of the loss of one cow during the final period.

The 3-day period mean daily milk yield and the butterfat percentage of the udder halves throughout the experiment are shown in Fig. 3. The mean daily milk yield and butterfat percentage of the udder halves in the pre-experimental, experimental, and post-experimental periods are given in Table 1. The difference between the udder halves in the experimental period is shown both uncorrected and corrected

for differences in the pre-experimental period. The individual responses of the six cows during the experiment are shown in Fig. 4.

An analysis of variance of the mean daily 3-day period milk yields during the experimental period is given in Table 2. The milk yields of the udder halves milked three times a day have been corrected for pre-treatment differences in yield between the udder halves.

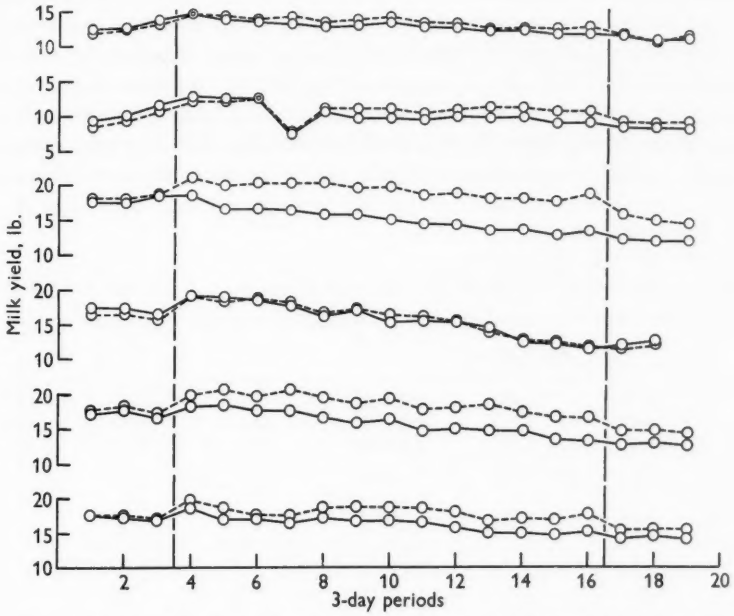

Fig. 4. Mean daily milk yield of udder halves in 3-day periods. Individual cows, Experiment 1. O—O, Twice a day milking; O···O, three times a day milking.

Table 2. *Analysis of variance of the milk yield in the experimental period*

	Degrees of freedom	Mean squares	*F* test: Error (1)	*F* test: Error (2)
Cows	5	173·17	***	***
Periods	12	18·86	***	***
Treatments	1	137·61	***	***
Cows × treatments	5	8·01	***	—
Periods × treatments	12	0·596	N.S.	
Error (1)	120	0·87	—	—
Total	155	—	—	—
Error + interactions (2)	137	1·11	—	—

Error, 6·1 %. *** Significant at the 0·1 % level; N.S., not significant.

Discussion

The udder halves which were milked three times a day significantly outyielded the halves which were milked twice a day, the increase in yield averaging 12 %. The butterfat percentage was unaffected throughout the experimental period.

The differences in yield were apparent on the first day of the experimental period, increased very quickly over the next few days, and then did not materially alter during the rest of the experimental period. The differences decreased during the 9-day post-experimental period. There was a significant difference between cows in response to the treatments, but all animals showed some increase in yield with three times a day milking.

To discover whether there was any difference in the amount of residual milk between the udder halves milked twice and three times a day, a comparison was made of the milk yield and butterfat percentage of the first milking 8 h after the treatment finished with the mean 8-hour milk yield and butterfat percentage of the last 3-day period of the experimental period. The udder halves which had been on 8-hourly milking should have given a similar yield after this first post-experimental 8-hour interval, but those which had been on 12-hourly milking should have out-yielded their mean 8-hour yield by the difference in residual milk after a 12-hour and an 8-hour interval.

Table 3. *Mean 8 h milk yield and butterfat percentage of the last 3 days of the experimental period, and of the first post-experimental milking*

	Milk per 8 h, lb			Fat, %		
	Left half (3×)	Right half (2×)	Difference in favour of right half	Left half (3×)	Right half (2×)	Difference in favour of right half
Last 3 days experimental	4·9	4·1	−0·8	3·9	3·8	−0·1
First milking post-experimental	5·0	4·3	−0·7	4·3	4·9	+0·6
Gain by right half	—	—	+0·1	—	—	+0·7

From the comparison given in Table 3 it would appear that the amount of residual milk was not greater with twice a day milking, but it had a higher butterfat content. This is in agreement with the results of Elliott (1961) where a comparison was made of the amount of residual milk and butterfat percentage after intervals between milkings of different lengths.

Experiment 2. The effect on yield and butterfat percentage of leaving a small extra amount of residual milk in the udder at each milking

The two treatments were normal milking and a milking which left approximately 1 lb more residual milk in the half-udder than the normal milking. This additional amount of residual milk was achieved by a delay of 6 min between stimulation of the udder and application of the milking machine (Elliott, 1961). Normal and delayed milking were applied to the same cow at the same time by using udder halves as the experimental units.

The animals used were the five Ayrshire heifers and one Shorthorn cow which had been on Latin square no. 2 in the preliminary experiment. They had been in milk 102 days on average and produced on average 16 lb of milk in 12 h at the beginning of the experiment, and 12 lb in 12 h at the end.

Any existing differences in yield between the halves were determined during a pre-experimental period of 9 days by twice daily milking. The experimental period lasted 39 days, during which time the animals were milked at 8-hour intervals. The left udder half of each cow was milked normally, and the right half milked 6 min later. The experimental period was followed by a further 9 days during which both halves were again milked normally twice daily (the post-experimental period). During the pre-experimental and post-experimental 9-day periods milking was carried out at 6 a.m. and 3 p.m. During the experimental period 8-hourly milking was practised, at 6 a.m., 2 p.m. and 10 p.m.

Immediately a cow entered the bail she began to feed. Without delay both udder halves were washed and a foremilk sample taken from each. As soon as this was finished, the cups were placed on the left half only. Six minutes later the other half-udder machine was placed on the right half. Both halves were stripped independently

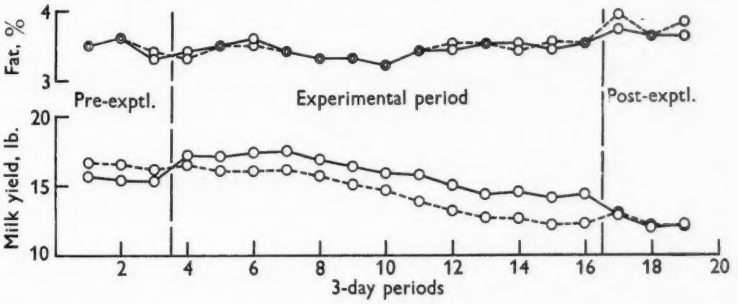

Fig. 5. Mean daily milk yield and butterfat percentage of udder halves in 3 day periods. Means of six cows, Experiment 2. O—O, Normal milking; O···O, delayed milking.

when ready, as judged by the Ruakura milk-flow indicators. In every instance the left half had been stripped and the machine removed before the 6 min delay had elapsed and the right half was due to be milked.

The milk yield from each half was recorded at every milking. Three-day composite butterfat samples were taken, a quantity of milk proportional to yield being taken at every milking.

The cows were kept in covered yards where they received a maintenance ration of hay and brewer's grains. Cattle cake was fed at the rate of 12 lb per day per cow, 4 lb being given at each of the three milkings.

Results

All milk yields are presented as mean yields in 3-day periods. Periods 1–3 inclusive comprise the pre-experimental period, 3–16 the experimental period, and 17–19 the post-experimental period.

The 3-day period mean daily milk yield and the butterfat percentage of the udder halves throughout the experiment are shown in Fig. 5. The mean daily milk yield and butterfat percentage of the udder halves in the pre-experimental, experimental, and post-experimental periods are given in Table 4. The difference between the udder halves in the experimental period is shown both uncorrected and corrected for

Table 4. *Mean daily milk yield and butterfat percentage of the udder halves*

	Left half (normal)	Right half (delayed)	Difference (decrease of right half)	Corrected difference	% corrected difference
Milk yield, lb					
Pre-experimental period	15·3	16·1	+0·8	0	—
Experimental period	15·9	14·3	−1·6	−2·4	15
Post-experimental period	12·3	12·4	+0·1	−0·7	6
Butterfat, %					
Pre-experimental period	3·5	3·5	0	—	—
Experimental period	4·1	3·9	−0·2	—	—
Post-experimental period	3·6	3·7	+0·1	—	—

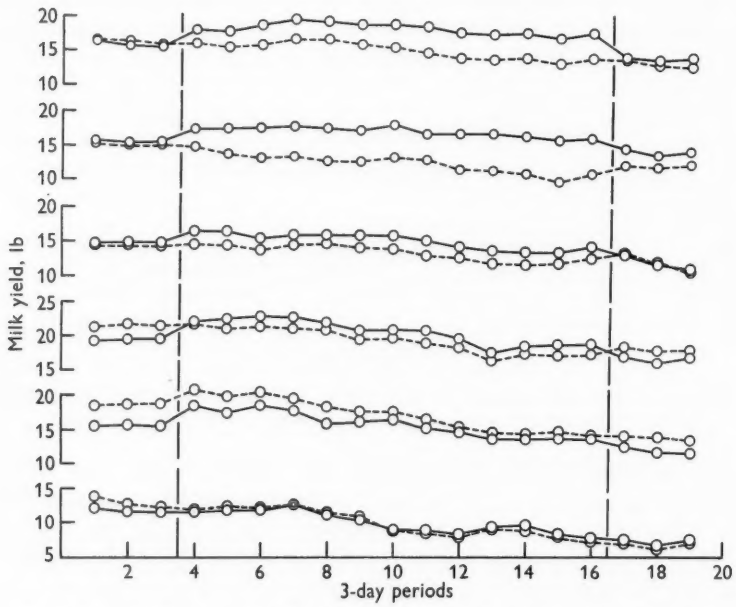

Fig. 6. Mean daily milk yield of udder halves in 3-day periods. Individual cows, Experiment 2. ○—○, Normal milking; ○ - - - ○, delayed milking.

Table 5. *Analysis of variance of the milk yield in the experimental period*

	Degrees of freedom	Mean squares	*F* test: Error (1)	*F* test: Error (2)
Cows	5	252·35	***	***
Periods	12	25·05	***	***
Treatments	1	255·13	***	***
Cows × treatments	5	11·24	***	—
Periods × treatments	12	0·56	N.S.	—
Error (1)	120	0·66	—	—
Total	155	—	—	—
Error + interactions (2)	137	1·03	—	—

Error, 5·5 %. *** Significant at the 0·1 % level; N.S., not significant.

differences in the pre-experimental period. The individual responses of the six cows during the experiment are shown in Fig. 6.

An analysis of variance of the mean daily 3-day period milk yields during the experimental period is given in Table 5. The milk yields of the udder halves milked after a delay have been corrected for pre-treatment differences in yield between the udder halves.

Discussion

The result of leaving a small amount of extra residual milk in one half-udder of each of the six cows at every milking was to reduce the average yield of those halves by 15% over a 39-day treatment period. Both milk and butterfat yields were similarly and significantly reduced by this amount, there being no effect on butterfat percentage.

The difference in yield increased throughout the experiment and reached 22%. A partial recovery of the affected halves took place during the 9-day post-experimental period.

To discover the difference in the amount of residual milk between the udder halves, a comparison of the yield and butterfat percentage of the first milking after the treatment finished with the average during the last 3 days of the experimental period was made (Table 6). The extent of the difference in the amount of residual milk between the udder halves milked normally and those milked after a delay should be shown by the increase in yield at the first post-experimental milking of the halves previously milked after a delay. The data in Table 6 shows that the 6 min delay was successful in causing the retention on average of an extra 1 lb of residual milk per half-udder.

Table 6. *Mean 8h milk yield and butterfat percentage of the last 3 days of the experimental period and of the first post-experimental milking*

	8 h yield, lb			Butterfat, %		
	Left half (normal)	Right half (delayed)	Difference in favour of right half	Left half (normal)	Right half (delayed)	Difference in favour of right half
Last 3 days experimental	4·8	4·1	−0·7	3·5	3·5	0
First milking post-experimental	5·5	5·8	+0·3	4·3	5·0	+0·7
Gain by right half	—	—	+1·0	—	—	+0·7

There was a significant difference in response between cows, but all animals were affected in some degree by the treatment. A comparison of the individual results together with the individual milk ejection response and extent of residual milk retention is presented in Table 7. The milk ejection response to the normal udder stimulation at the beginning of milking was measured four times for each cow during the experiment by means of $\frac{1}{2}$-min milk yield recordings during milking. This response is presented in the Table as the number of times it failed to occur at these four milkings.

This comparison shows that there is a definite connexion between the success of the treatment in causing an increase in residual milk and the lowering of yield. Of the three cows showing least effect, no. 59, whose milk ejection appeared to be readily stimulated by milking the normally milked half-udder, very often had a second ejection during milking of the delayed half. The other two, nos. 11 and 55, showed the greatest number of milk ejection failures. Ejection occurred either later or not until the milking of the delayed half was begun. The cow showing the greatest effect of the delayed milking (no. 58) was observed throughout the experiment to have an excellent ejection and never a second response at the delayed milking.

Table 7. *Connexion between effect of treatment and success of treatment in causing a residual milk retention*

	Cow no.					
	11	52	53	55	58	59
Percentage decrease in yield of delayed half	13	20	16	5	26	10
Number of milk ejection failures in four milkings	3	0	2	3	0	1
Gain in milk yield of delayed half at first post-experimental milking	0·1	1·0	1·7	0·7	1·9	0·6

GENERAL DISCUSSION AND CONCLUSIONS

In these experiments three times a day milking compared with twice a day milking caused an increase in milk yield of 12%, and inefficient milking leading to the retention of an extra 1 lb of residual milk per half-udder caused a decrease of 15%. It is not possible to eliminate with certainty differences in milk accumulation in the udder as the cause of these differences in yield. There may be a long-term indirect effect of milk accumulation that is undetectable over as short a period as was used in the preliminary experiment to determine its effect on milk secretion rate.

In Experiment 1, where twice and three times a day milking were compared, the increase with three times a day milking could not have been due to any immediate effect of relief of milk accumulation in the udder, for the absence of any immediate benefit had been shown in the preliminary experiment at these intervals and yields. Neither can it be due to the more frequent release of oxytocic hormone, as both experimental units belonged to the same cow and would suffer no such difference. A third possible cause is the differences in the amount of residual milk. From the work of Elliott (1961) and from Experiment 1 itself, these appear not to be very great, and to exist mainly in the form of differential butterfat retention. It is impossible to say whether the smaller butterfat content of the residual milk after the 8 h interval compared with the 12 h interval could have as great an effect on yield as that obtained. It can only be suggested that a significant effect of the treatment was obtained; that it is not due to either the immediate effects of relief of milk accumulation or to any difference in oxytocic hormone level; and that it is perhaps due to some local effect of the treatment on the secretory cells, caused either by the consistently smaller accumulation of milk between each milking or by the retention of a residual milk of lesser butterfat percentage at the end of every milking.

Very similar conclusions can be drawn from Experiment 2 where the delayed milking treatment caused a decrease in milk yield. This effect, again, cannot be due to any immediate effect from the greater milk accumulation or to any difference in the level of oxytocin. Again it can be suggested that the effect is a local one in the udder cells, but in this case the evidence is stronger as there was a considerable difference in the amount of residual milk.

In both Experiments 1 and 2 it might be considered possible to extract more information regarding the cause of the treatment effect from the fact that there was an immediate partial recovery the moment the treatment was discontinued, and from the fact that the treatment effect reached its maximum very quickly for one treatment but increased throughout the experimental period for the other. The partial recovery would indicate that some at least of the effect is not due to irreversible deterioration of the cells. The difference in speed of response could indicate that the cause is different for the two treatments, being immediate for one and cumulative for the other. An immediate effect, as in the comparison of twice and three times a day milking, does not suggest gradual loss of cell efficacy, but rather something akin to the effect of long intervals (see Elliott *et al.* 1960). The gradual and continuous adverse effect, as experienced in the comparison of efficient and inefficient milking, is far more suggestive of an effect on cell efficiency.

I wish to thank Dr F. H. Dodd for his most valuable help and advice.

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A comparison of the influence of *Streptococcus lactis* and *Str. cremoris* starters on the flavour of Cheddar cheese

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Summary. Comparable cheeses were made using single strains of *Streptococcus cremoris* and *Str. lactis* as starters. The three *Str. lactis* strains imparted a characteristic rather abnormal flavour to the cheese, whereas the three strains of *Str. cremoris* all gave cheeses of normal flavour. The abnormal flavour caused by the lactis cultures increased in intensity as ripening proceeded, and differed slightly from strain to strain.

Bacteriophage usually developed slightly in the whey and in the curd during the manufacturing process, but never to such a degree as to delay acid production unduly. The extent of phage development did not have a significant influence on cheese flavour.

All the lactis strains survived in much larger numbers in the cheese than the cremoris strains. The simplest explanation of the data is that the 'lactis' flavour was a direct effect, due to flavour substances produced by the *Str. lactis* cells present in the young cheese.

The question whether the lactic streptococci present in cheese starters have a significant influence on the flavour of cheese has been debated and investigated for many years. Early workers (e.g. Hucker & Marquardt, 1926) failed to realize that the flavour of cheese depends very largely on the pH of the curd and, to a lesser degree, on its moisture and salt contents. In the earlier work, many of the differences in flavour attributed to the influence of added bacteria must have been due to differences in the pH of the cheese at the beginning of the ripening period. However, by using starters of known constant activity, the moisture and salt contents of the cheese and its pH can all be controlled fairly closely. Under such conditions, although the adventitious microflora remains as an uncontrolled variable, it is reasonable to assume that any flavour difference which always occurs between cheeses made with different pure culture starters is due directly or indirectly to a difference in the biochemical characteristics of the starter bacteria being compared.

Kelly (1932*a*, *b*) concluded that cheeses made with single strains of *Str. lactis* and *Str. cremoris* as starters developed desirable flavours just as well as those cheeses made with commercial starters containing various streptococci and leuconostocs. The same conclusion was reached in early work at this Institute. It was confirmed by application in commercial practice in this country, and for more than 20 years

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most New Zealand Cheddar cheese has been made using starters consisting of single strains of *Str. lactis* or *Str. cremoris*. These are maintained separately in pure culture and a pair of such single strains is generally used for cheesemaking. Strains of *Str. cremoris* have been preferred because of the greater strain specificity of the bacteriophages which attack them.

The only report of a flavour difference associated with single strain (group N) starters relates to bitterness in cheeses made with certain strains (Emmons, McGugan & Elliott, 1960; also personal communication). Some strains never, while others almost always, produced the defect. With the remainder, however, the development of bitterness depended on the pH of the cheese curd, no bitterness being detectable unless the pH had been less than a certain critical value.

Recently an abnormal flavour, which was described by graders as 'dirty' or 'fruity', was noticed in some of the mature experimental cheeses made at this Institute. It was found that this particular flavour developed in cheeses made with starter ML_3 (a strain of *Str. lactis*), alone or in combination with other strains. It was noted, too, that in such cheeses the pH values at 6 months had risen more from the 14-day values than had the pH of most other cheeses. These observations prompted a further comparison of various *Str. lactis* and *Str. cremoris* strains available for use as starters.

EXPERIMENTAL

Method of manufacture

Bulked milk from five predominantly Jersey herds was used. The milk was of high bacteriological quality, methylene blue reduction times always exceeding 6 h. The milk was flash-pasteurized at 150 °F and the cheeses made in 80-gal vats. By making modifications to the schedule during the cheesemaking process it was hoped to produce cheese possessing (at 14 days): (i) a pH of 5·0–5·1, (ii) about 54 % of water in the non-fatty substance (W.N.F.S.) and (iii) 4–5 % of salt in the cheese moisture. The experiments were carried out at intervals over the course of 6 months and, in consequence, the composition of the milk varied considerably in the different trials. The aim of the cheesemaker was to hold the W.N.F.S. steady rather than the moisture content of the whole cheese, since a steady value for W.N.F.S. was considered the more important (Whitehead, 1948). The curd from each vat was made into an 80 lb cylindrical cheese and kept at 55 °F and 80–85 % r.h. for 2 weeks. After drawing plugs for analysis the cheeses were waxed and stored at 45 °F, 80–85 % r.h.

Starter bacteria

Three strains of *Str. lactis* (ML_2, ML_3, ML_8) and three of *Str. cremoris* (C_{13}, KH, HP) were used. Fourteen experiments were carried out, in each of which a comparison of one of the *Str. lactis* with one of the *Str. cremoris* strains was made under identical conditions of cheese manufacture, strain HP being used in most of the comparisons. On two occasions an extra cheese was made with a starter consisting of equal volumes of *Str. cremoris* and *Str. lactis* starters added to the vat.

Estimation of bacterial population and bacteriophage concentration

Bacteria in milk

Immediately before and 5 min after adding the starter a 250 ml sample of the milk was taken from the vat. A 10 ml portion was homogenized in 90 ml of quarter-strength Ringer's solution for 2·0 min using an MSE Ato-mix blender. The homogenate was allowed to stand for 10 min and decimal dilutions made in Ringer's solution. Using 1 ml portions of suitable dilutions as inocula two series of plates were prepared. One series was treated in the usual manner, using lactose yeast phosphate agar (Hunter, 1946) as the growth medium; from this series, counts of total population were obtained. Counts of the non-starter population were found from the second series by the method of Robertson (1960), using phage prepared according to Whitehead & Bush (1957). To determine the proportions of fast and slow starter substrains, and in the later samples, when the difference between total and non-starter counts had become small, to determine the proportions of starter and non-starter bacteria present, fifty adjacent colonies were picked into yeast litmus milk from a suitable plate of the total count series. The cultures were incubated at 30 °C, inspected for acid production after 24 h, and cultures examined microscopically after 3 days.

Bacteria in cheese

Samples were taken at 'dressing', i.e. when the cheese had been in the press for 30 min, and at 1, 3, 5, 9, 14 and 26 weeks. Cores were taken vertically at dressing and radially thereafter. A 10 g portion, taken from that part of the core nearest to the centre of the cheese, was homogenized in quarter-strength Ringer's solution and the emulsion treated as described for milk samples.

Bacteriophage

Coagulated milk cultures of the appropriate starters were spread on lactose yeast phosphate agar and spotted with one standard drop (0·004 ml) of each dilution of the series prepared for the bacterial counts. Decimal dilutions in quarter-strength Ringer's solution were prepared from samples of whey taken at running, and treated similarly.

RESULTS

Composition of the cheeses

In the comparison of each pair of single strain starters the cheesemaker attempted to control operations in the two vats so as to maintain strict equivalence at all stages of the manufacturing process (see Table 1) and thus to produce cheeses which were closely comparable in W.N.F.S., pH and % salt in the cheese moisture. Results of the cheese analyses are given in Table 2. The values for fat in the water-free substance (F.W.F.S.) depend, of course, on the composition of the original milk, and are not under the control of the cheesemaker. Hence the range of variation in F.W.F.S. was inevitable and expected, since the milk varied in composition during the experimental period. The other fat and moisture values depended on the expulsion of moisture during manufacture through such agencies as acidity, temperature,

mechanical stirring and salt. Table 2 shows that the most important aim, namely, that of producing a pH of 5·0–5·1 in the cheeses when a fortnight old, was in fact achieved. The more difficult objectives of 54 % W.N.F.S. and 4–5 % salt in the cheese moisture were less successfully achieved. The range of W.N.F.S. values was about equal to that of commercial cheeses, but the variation in % salt in the cheese moisture was greater than had been expected. The extreme values were for two lactis cheeses, both with 6·3 % salt in the cheese moisture and with W.N.F.S. values of 52·0 and 52·4 %.

Flavour of the cheeses

The cheeses were examined by a panel of four or five trained observers at 14 days and thereafter at intervals of 2 weeks and later of 4 weeks, until they were 6 months old. The members of the panel worked together while examining the samples, but the origin of the individual cheeses was unknown to them. The cheeses made with cremoris strains gradually developed a normal cheese flavour, with variations in intensity from cheese to cheese in the usual random manner, but the lactis cheeses all possessed, even at 14 days, an unusual flavour superimposed on the normal Cheddar flavour. In the early stages of ripening this unusual flavour was by no means objectionable, and might not have been remarked on had not the corresponding cremoris cheese been available for comparison. It was variously described as 'dirty', 'fruity', or merely as 'off-flavour', and is now termed 'lactis' flavour in this laboratory. It usually became more pronounced with maturity. 'Lactis' flavour varied according to the starter strain present and some observers could successfully name the strain used from the flavour of the cheese. Cheeses made using strain ML_3 always possessed a stronger 'lactis' flavour than cheeses of corresponding ages made with either ML_2 or ML_8.

The differences in flavour between the cremoris and lactis cheeses were observed throughout the whole series of experimental comparisons, in spite of the variations in composition noted above. These variations in composition might have been expected to have had an influence on flavour, especially where high salt content was concerned.

Bacteriophage

The numbers of phage particles in the milks at rennetting, in the wheys at running and in the cheeses during the ripening period were determined in eight of the comparisons. The results are given in Table 3. Phage was detected in eleven of the sixteen cheeses; of the 'phage-free' cheeses, two had been made with strain ML_8 as starter and one each using strains C_{13}, HP and ML_2. Since care was taken to arrange that the starters used in the experiments were kept out of use for normal cheese manufacture for several days before an experiment, the concentration of phage in the factory capable of attacking any experimental starter strain should have been low. Nevertheless, in most instances it was obviously sufficient to lead to infection of the starter in the cheese vat. It is interesting to note here that phage for starter strain ML_8, the only phage undetected on more than one occasion in this series, falls sixteenth in a descending scale of the multiplication rates of the phages active against the seventeen single strain starters in regular use in the Institute factory (Whitehead, personal communication).

Table 1. *Manufacturing data*

(80 gal flash-pasteurized milk; 1½% single strain starter; 80 ml rennet)

	Str. lactis (14 cheeses)		*Str. cremoris* (14 cheeses)		Differences within pairs of cheeses*	
	Average	Range	Average	Range	Average	Range
% acidity at rennetting	0·16	0·15–0·17	0·16	0·15–0·18	0·00	−0·01–0
% acidity at cutting	0·11	0·09–0·11	0·11	0·10–0·11	0·00	−0·01–+0·01
% acidity at running	0·14	0·14–0·15	0·14	0·14–0·15	0·00	0–+0·01
% acidity at drying	0·16	0·15–0·17	0·16	0·15–0·16	0·00	0–+0·01
% acidity at milling	0·49	0·40–0·53	0·49	0·45–0·51	0·00	−0·05–+0·04
% acidity at salting	0·72	0·64–0·80	0·70	0·65–0·75	+0·01	−0·04–+0·05
Time, rennetting to drying h and min	2.36	2.20–3.05	2.35	2.20–2.55	+0.01	−0.20–+0.15
Time, drying to salting, h and min	2.38	2.05–3.20	2.35	2.25–2.45	+0.02	−0.30–+0.40
Total making time, h and min	5.14	4.40–6.05	5.10	4.40–5.30	+0.03	−0.50–+0.55

* + indicates value for lactis cheese greater than for cremoris cheese of the same pair.

Table 2. *Analyses of cheeses at* 14 *days*

(80 gal flash-pasteurized milk; 1½% single strain starter; 80 ml rennet)

Analysis at 14 days	*Str. lactis* (14 cheeses)		*Str. cremoris* (14 cheeses)		Differences within pairs of cheeses*	
	Average	Range	Average	Range	Average	Range
Moisture, %	33·8	32·1–35·3	34·6	33·4–36·2	−0·8	−1·8–+0·2
Fat, %	37·4	35·9–38·8	36·9	35·5–38·0	+0·4	−0·1–+0·8
F.W.F.S., %	56·4	55·3–57·7	56·4	55·3–57·7	0·0	0·0
W.N.F.S., %	54·0	52·0–55·6	54·8	53·5–56·7	−0·6	−1·6–+0·2
Salt, %	1·7	1·3–2·0	1·6	1·3–1·7	+0·1	−0·1–+0·3
Salt in the cheese moisture, %	4·9	3·8–6·3	4·5	3·8–5·1	+0·3	−0·2–+1·2
pH	5·0	5·0–5·1	5·0	5·0–5·1	0·0	−0·1–+0·1

* + indicates value for lactis cheese greater than for cremoris cheese of the same pair.

In normal cheesemaking it often happens that there is some phage development without serious slowing of acid production, so that phage is present in the cheese. Thompson (1959) has shown that the activity of phage is inhibited at specific levels of acidity depending on the particular starter strains. This also occurs in cheese: it may be seen from Table 3 that the concentration of phage did not increase as the cheeses ripened. During the plating of cheese samples, however, the neutral conditions of the emulsifying and diluting solutions would allow absorption, and subsequently any bacterial cells which had been attacked would lyse in the plates although new phage particles would be unlikely to reach uninfected bacteria growing some distance away as they would be on countable plates (Thompson, 1959). Thus

Table 3. *Numbers of phage particles present in sixteen single strain starter cheeses during ripening*

		No. of phage particles, millions						
				Cheese,/g				
Starter	Date of manufacture of cheese	Milk, /ml	Whey, /ml	At dressing	At 1 week	At 3 weeks	At 5 weeks	At 26 weeks
Str. lactis								
ML_2	17. viii. 59	*	0·1	0·1	0·1	0·1	0·1	0·1
ML_2	9. xi. 59	*	0·1	10	10	10	10	1
ML_2	29. i. 60	*	*	*	*	*	*	*
ML_3	4. viii. 59	*	0·1	10	10	10	1	1
ML_3	12. xi. 59	*	0·01	10	1	0·1	0·1	0·1
ML_3	15. i. 60	*	*	0·1	0·1	0·01	0·1	0·1
ML_8	12. viii. 59	*	*	*	*	*	*	*
ML_8	22. i. 60	*	*	*	*	*	*	*
Str. cremoris								
C_{13}	17. viii. 59	*	*	*	*	*	*	*
C_{13}	9. xi. 59	*	0·1	10	1	10	1	10
C_{13}	29. i. 60	*	0·01	1	1	1	0·1	1
HP	4. viii. 59	*	*	*	*	*	*	*
HP	12. xi. 59	*	1	1	0·1	0·1	0·1	0·01
HP	15. i. 60	*	0·01	1	1	0·01	0·1	0·01
KH	12. viii. 59	*	10	100	100	100	100	100
KH	22. i. 60	*	... 100	100	100	100	100	100

* < 1 particle/standard drop (0·004 ml) of 10^{-1} cheese dilution, i.e. < $0{\cdot}0025 \times 10^8$ particles/g or /ml.

when low numbers of phage particles relative to the numbers of streptococci were found, it has been assumed that the counts of the starter organisms have not been seriously affected by the presence of phage. However, the starter counts for both of the cheeses made with strain KH have not been included when averaging the results of bacterial counts, as phage kh was present in relatively high concentrations. The actual concentrations of phage in the experimental cheeses had no apparent influence on the development of flavour. This accords with commercial experience where it is often observed that cheeses in which a high concentration of phage is present develop flavours as desirable as those of cheeses in which phage cannot be detected.

Bacterial flora

Bacterial counts of milk, curd and cheese at various ages up to 26 weeks were made in eight of the experiments comparing lactis and cremoris cheeses. The results are not given in detail, since the main finding was that the *Str. lactis* strains survived

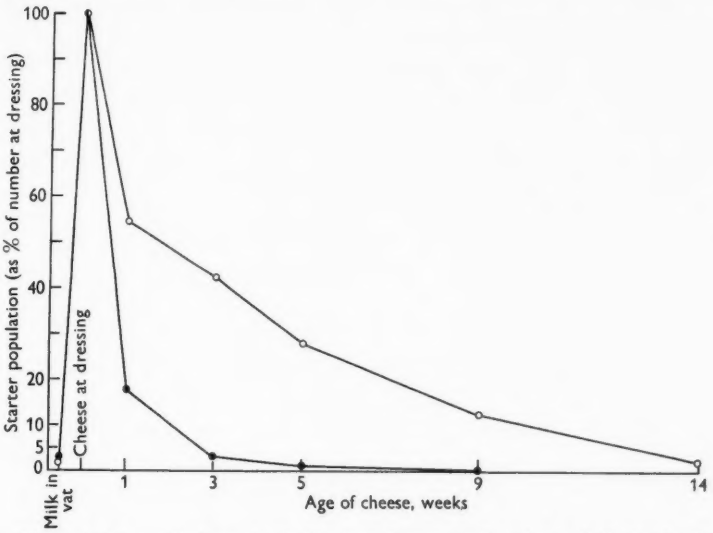

Fig. 1. Mean starter survival curves for fourteen cheeses made using a single strain of either *Str. lactis* or *Str. cremoris*. ○—○, *Str. lactis*; ●—●, *Str. cremoris*.

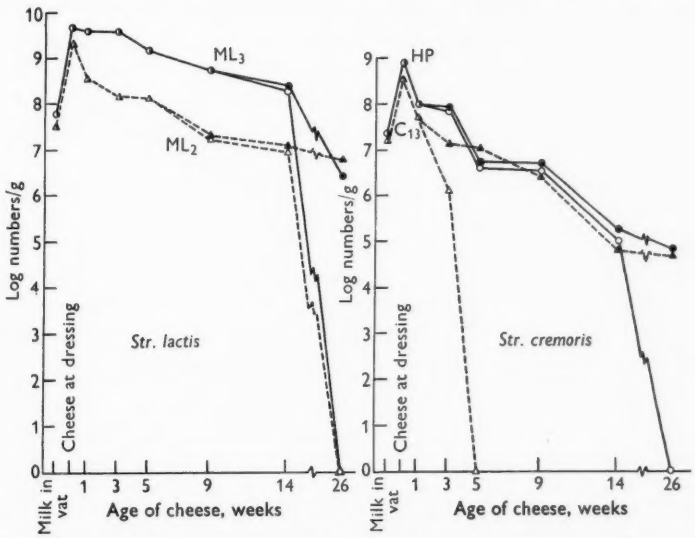

Fig. 2. Starter and total population trends in four of the fourteen cheeses made using a single strain of either *Str. lactis* or *Str. cremoris*. ○—○, Starter counts in the cheese showing highest starter population; ●—●, total counts in that cheese; △ - - -△, starter counts in cheese showing lowest starter population; ▲- - -▲, total counts in that cheese.

in much larger numbers in the cheese than did the *Str. cremoris* strains. This confirms the results of Dawson & Feagan (1957). The average survival trends for the two species are illustrated in Fig. 1 (two cheeses made with strain KH excluded). In preparing these curves the numbers of starter streptococci present at various ages in the ripening cheeses were first calculated as percentages of the respective starter populations at the end of the cheesemaking process, then the average plotted for each species. This method of presentation compensates for the wide differences in absolute numbers of the two species present in the cheese curd, and illustrates differences in survival of *Str. lactis* and *Str. cremoris* strains more clearly than does the semi-logarithmic presentation of Dawson & Feagan (1957). Fig. 1 shows that, in general, after 3 weeks' ripening the starter represented less than 5 % of the initial numbers in the cremoris cheeses, whereas in the lactis cheeses it was still 42 % at 3 weeks, and only fell to 5 % after more than 12 weeks. The range of starter and total counts for the two types of cheese is shown in Fig. 2. Again the counts of cheeses made with strain KH have been excluded. Total counts of the milk before adding the starter were ever greater than 10^4/ml.

pH *of the cheeses*

The observations on the small number of cheeses which stimulated the present investigation had indicated that in cheeses made with *Str. lactis* as starter the pH values rose more as ripening proceeded than in cheeses made with *Str. cremoris*. Determination of the pH value of the present cheeses at 2 and 26 weeks showed no marked differences in change of pH during ripening between the two types of cheese.

DISCUSSION

The observation that cheeses made with *Str. lactis* as starter develop an abnormal flavour as compared with cheeses made with *Str. cremoris* shows that starter streptococci can influence cheese to give a flavour distinct from that described by Emmons *et al.* (1960). Both lactis and cremoris strains are sometimes present in mixed commercial starters, often with one or other species comprising the major part of the culture.

As it has been shown also that strains of *Str. lactis* survive longer in cheese than do *Str. cremoris* strains, it is of interest to know whether the 'lactis' flavour is a direct effect, due to the production of some flavouring substance(s) by *Str. lactis*, or an indirect effect, caused by the suppression (due to the longer survival of *Str. lactis*) of those adventitious bacteria which would otherwise have proliferated and caused the more normal flavour to develop. The evidence seems to be in favour of the former explanation, since the characteristic flavour of cheeses made with *Str. lactis* is detectable as an addition to normal cheese flavour and increases as ripening proceeds. Furthermore, there are slight but distinct differences in the abnormal flavour in cheeses made using different strains of *Str. lactis*. It is suggested that, although unable to proliferate in cheese, the resting cells of *Str. lactis* produce metabolites which impart the characteristic flavour distinguishing lactis cheeses from those made with *Str. cremoris*. It is possible that the observed difference in flavour of the lactis and cremoris cheeses was due to stimulation of flavour-producing adventitious bacteria by the longer surviving *Str. lactis* cells, but this seems unlikely.

Finally, it should be emphasized that the cheeses were of good body, texture and flavour, were manufactured under standard conditions and were within the normal range of chemical composition and pH. Analytical and manufacturing data have been included since it is difficult, if not impossible, to assess the significance of bacteriological findings unless such information is given.

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The effect of level of concentrate feeding before and after calving on the production of dairy cows

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Summary. Two levels of steaming-up and two levels of concentrate feeding after calving were compared in a feeding trial extending over a complete lactation using sixteen autumn-calving Ayrshire cows in each of 2 years. On the high and low levels of steaming-up 3·3 and 1·6 cwt of concentrates respectively were fed per cow in the 5 weeks before calving. On the high and low levels of concentrate feeding after calving the concentrates were fed at rates of 4·1 and 2·2 lb per 10 lb of milk respectively from the third day after calving until spring grazing began. The total weight of concentrates fed after calving was 25·8 cwt per cow on the high treatment and 12·1 cwt on the low treatment. All the cows grazed good pasture in the autumn and the following spring and had a ration of 9–11 lb of hay and 50–90 lb of grass silage per day during the winter feeding period. During this period the estimated mean intakes of starch equivalent on the high and low concentrate treatments were 17 and 13 lb/day, which were equivalent to 122 and 103 % of Woodman's standards respectively.

Milk yield, milk quality, length of lactation and live weight were not affected by the difference between the steaming-up treatments, and no interaction between steaming-up and post-calving treatments was found. Average milk yields in the first 70 and 140 days after calving and in the complete lactations were all higher on the high post-calving concentrate treatment than on the low treatment, and the response per 1 lb additional starch equivalent fed was 1·05 lb milk. The fat content of the milk was similar on all treatments whereas the S.N.F. content increased from 8·64 % on the low treatment to 8·82 % on the high treatment, giving a response of 0·045 % S.N.F. per 1 lb of additional starch equivalent.

Larger losses in live weight occurred on the low treatment in the first 10 weeks after calving than on the high treatment, but in both groups the weights were almost identical after 45 weeks.

During the last few years several winter feeding experiments with dairy cattle have been conducted at this Institute to investigate the effect of feeding different amounts of concentrates on the yield and quality of milk (Reid & Holmes, 1956; Holmes, Reid, MacLusky, Waite & Watson, 1957; Castle, MacLusky, Waite & Watson, 1958; Castle, MacLusky, Morrison & Watson, 1959). In each of these trials four rations were compared and the basic experimental design was four consecutive feeding periods each of 3 or 4 weeks, the cows being allocated at random to the treatments in a balanced Latin square layout. At the beginning of the experiments

the cows were always in the declining phase of lactation and, in general, the experimental error was low and it was possible to detect small differences between treatments. The results of the trials, however, applied only to one part of the lactation and as Broster (1958) has stated, milk production must be studied in terms of the lactation as a whole and not solely as an instantaneous input/output relationship. Furthermore, it is now known that response in milk yield to given amounts of additional feed varies with the stage of lactation (Blaxter, 1959). The effect of different levels of concentrate feeding over an entire lactation has been studied in America by Jensen, Klein, Rauchenstein, Woodward & Smith (1942) but little of a similar nature has been done in this country. The recent study of Broster, Ridler & Foot (1958) compared milk yields, milk composition and live weight changes throughout lactation, but the contrasting levels of concentrate feeding were given only for the last 21 days of pregnancy and the first 84 days of lactation, after which all the animals were given the same treatment. In the trial which is described below a basal diet of hay and grass silage was supplemented with two levels of concentrate feeding for 5 weeks before calving and for up to 30 weeks after calving.

EXPERIMENTAL

The experiment was conducted for two consecutive periods each of approximately 12 months from July 1957 to August 1959 and in each of the 2 years twelve Ayrshire cows and four first-calf heifers in the Institute herd were used, thirty-two animals in all. Each year the cattle were divided into four groups of four, and the animals in each group were matched as nearly as possible in potential milk yield as judged by previous lactation records, peak yield in the previous lactation, live weight and date of calving.

Feeding and nutrient value of feeds

The four animals in each group were allocated at random to one of four concentrate feeding treatments as follows:

Treatment	Level of concentrate feeding	
	Before calving	After calving
High–high (HH)	High	High
High–low (HL)	High	Low
Low–high (LH)	Low	High
Low–low (LL)	Low	Low

The weight of concentrates fed on the two steaming-up treatments before calving were:

Treatment	Concentrates, lb/day, in weeks before calving				
	5	4	3	2	1
High	4	8	12	12	12
Low	2	4	6	6	6

The weight of concentrates fed after calving were:

High	4 lb/10 lb milk
Low	2 lb/10 lb milk

In addition 2 lb of concentrates/day were fed to each animal on the high treatment and 1 lb/day on the low treatment until the peak yield of the lactation was reached. On the day of calving and the following day no concentrates were fed. The amount given was adjusted on Mondays, Wednesdays and Fridays until the peak milk yield was reached, after which the adjustment was made once per week. Half the weight of concentrates was fed at each milking time. The ingredients and the composition of the concentrate mixture are given in Table 1.

Table 1. *The composition and calculated feeding value of the concentrate mixture and the roughages* 1957–58 *and* 1958–59

The concentrate mixture

	Parts by weight	Composition as fed	1957–58	1958–59
Oats (bruised)	4			
Barley (ground)	2	Dry matter, %	84·4	84·3
Flaked maize	1	Approximate starch equivalent	66	66
Soya bean meal	1			
Locust bean meal	½	Approximate digestible crude protein, %	12	13
Tic bean meal	½			
Groundnut cake	½	Crude protein (by analysis), %	14·4	15·6
Grass meal (ground)	½			

The roughages

	Hay			Grass silage	
	1957–58	1958–59		1957–58	1958–59
		1	2*		
Dry matter, %	85·4	85·4	82·8	24·5	21·3
Crude protein of dry matter (by analysis), %	7·8	7·0	5·2	12·4	11·2
Starch equivalent	34	32	30	12	11
Digestible crude protein, %	3	3	2	2·0	1·5

* Fed after 15 Feb. 1959.

The other feeds given to the cows varied according to the time of year. In both seasons, first-class leafy pasture was grazed by all the animals from the date when the steaming-up of the first animal began, 26 July 1957 and 28 July 1958 until the winter feeding programme started. The herbage contained an average of 15·2 and 15·4 % crude protein in its dry matter in the late summer and autumn of 1957 and 1958 respectively. In 1957 the cattle were housed at night for the first time and fed hay on 21 October, but in 1958 this was not necessary until 31 October. In addition after these dates kale was grazed during the day for 2–3 weeks and the regular winter diet of hay and grass silage did not begin until 11 November 1957 and 8 December 1958. Spring grazing began on 28 April 1958 and 4 April 1959. When at pasture in the spring the animals did not receive concentrates or any other supplementary feedingstuffs.

During the winter feeding period 9–11 lb of hay was offered to each animal at 5 p.m. and grass silage at 7 a.m. and 12 noon. The silage, 50–90 lb per cow per day, was offered in sufficient amounts so that a measurable residue was always left in the

feeding troughs. All hay and silage were individually weighed when fed to the cows and on 5 days in each week feed refusals were weighed.

The silage was made in the same silo each year and had a mean dry-matter content of 24·5 % in 1957–58 and 21·3 % in 1958–59 (Table 1). This difference is attributed to the fact that although the herbage ensiled was at a similar stage of growth in both years, the silage in 1957 was made under ideal conditions. In contrast, in 1958, 0·64 in. of rain fell during silage making. The grass was cut in late May at the long leafy stage and no preservative was added. The hay was made from somewhat more mature herbage cut at the early flowering stage and consisted largely of Italian ryegrass with a little perennial ryegrass. The starch equivalent and digestible crude-protein content of the concentrate mixture were calculated from Evans (1960), and the hay and silage values from Moon (1954) and McDonald & Purves (1956) respectively.

During the winter period the cows were kept in a conventional byre and allowed a period of approximately 2 h daily for exercise in a bare paddock.

Milk yield and quality

The cows were milked twice a day and their yields recorded at each milking to the nearest $\frac{1}{4}$ lb. Any animal which yielded less than 56 lb of milk in 7 consecutive days at the end of a lactation was immediately dried off. Milk samples for the determination of fat and solids-not-fat were taken from all the cows each week on Wednesday night and Thursday morning, and were mixed in proportion to yield to give a 24-hour sample for each cow. The fat content was determined by the Gerber test (British Standards Institution, 1955) and the total solids content gravimetrically. To detect any mastitis that might occur, samples of foremilk were taken every 2 weeks and examined bacteriologically and by the differential cell-count method of Blackburn, Laing & Malcolm (1955). Quarters with abnormal cell counts or containing pathogenic bacteria were treated with antibiotics.

Live weight and services

The cows were weighed on Thursday morning each week from the time when steaming-up started until grazing began in the spring, and after that they were weighed once a month. In addition each cow was weighed a few hours after calving. All the cows were served at the first heat after a period of 10–12 weeks from calving.

RESULTS

All the cows completed their lactations without any major mishap, and the small ailments which occurred had no serious effect on milk yields.

Feed consumption

Concentrates

The amounts of concentrates fed on the different treatments are given in Table 2. The weights fed before calving were 3·3 cwt and 1·6 cwt/cow on the high and low steaming-up treatments respectively. After calving the cows on the high lactation feeding received 25·8 cwt and the ones on the low lactation feeding received 12·1 cwt.

The animals on the HH treatment consumed a total of 29·8 cwt/cow before and after calving, which was more than twice the amount eaten by the cows in the LL group.

No concentrates were fed when the cows were at pasture in the spring, so the amount of concentrates fed after calving per 10 lb of milk produced was calculated in two ways (Table 2). Over the first 140 days of lactation all cows received concentrates and the average consumption was 4·1 and 2·2 lb per 10 lb milk for the high and low treatments respectively, whereas when calculated for all the milk produced in the lactation the corresponding values were 3·4 lb and 1·8 lb.

Table 2. *The weight of concentrates fed per cow and per 10 lb milk*

	Treatment				Mean amounts fed to the cows that were on the high and low treatments before calving		Mean amounts fed to the cows that were on the high and low treatments during lactation	
	HH	HL	LH	LL	High	Low	High	Low
Total concentrates fed, cwt								
Before calving	3·3	3·3	1·6	1·6	3·3	1·6	2·4	2·4
During lactation	26·5	11·7	25·1	12·4	19·1	18·7	25·8	12·1
Total	29·8	15·0	26·7	14·0	22·4	20·3	28·2	14·5
Concentrates fed during lactation, lb/10 lb of milk								
Days 1–140	4·10	2·20	4·15	2·18	—	—	4·12	2·19
All lactation	3·46	1·79	3·42	1·80	—	—	3·44	1·80

Dry matter

The mean daily consumption of dry matter of each cow was calculated for the winter period when the cows were housed and individual feeding of the animals was possible. The results were averaged for all the cows receiving the low concentrate treatment during lactation (groups HL and LL) and also for all the cows on the high concentrate treatments during lactation (HH and LH). The findings are summarized in Table 3. In both years the low level of concentrate feeding resulted in a lower level of total dry-matter intake than did the high level of feeding. When the daily dry-matter intakes were expressed as a percentage of live weight (Table 3) the differences between treatments were again clearly visible, particularly in the second winter of the experiment, 1958–59. On both concentrate treatments there was a lower mean intake of dry matter in the 1958–59 period than in the previous year. On the high treatment this was due to a lower intake of concentrate dry matter but on the low treatment the intakes of concentrates and of roughage dry matter were both lower in 1958–59 than in 1957–58.

Concentrate replacement

On the high treatment, for every 1 lb of concentrate dry matter consumed over the amount consumed on the low treatment, the average decline in the intake of roughage dry matter was 0·38 lb in 1957–58 and 0·18 lb in 1958–59. The concentrate replacement value in 1957–58 was similar to that obtained in other trials with

concentrates (Reid & Holmes, 1956; Holmes *et al.* 1957) and with roots (Castle, Drysdale & Waite, 1961), whereas the value obtained in 1958–59 was almost identical with the one found by Holmes, Arnold & Provan (1960).

Table 3. *The average consumption of dry matter and starch equivalent per cow on the high and low level of concentrate feeding during the winter months*

Winter feeding period	Level of concentrate feeding after calving	Mean dry-matter intake		Mean starch equivalent intake	
		lb/day	% of live weight	lb/day	% of Woodman std.*
1957–58	High	31·8	2·9	18	121
	Low	27·5	2·7	14	104
1958–59	High	29·2	2·7	16	122
	Low	24·0	2·3	12	102

* Evans (1960).

Silage dry matter intake

The mean weekly dry-matter content of the silage varied widely. In 1957–58 it ranged from 17·4 to 30·7 % and in 1958–59 from 17·7 to 26·6 %. These changes were closely related to changes in the total daily dry-matter intake of the cows, and to investigate this matter regressions of the mean daily silage dry-matter intake on the dry-matter content of the silage were calculated for each treatment. These showed that for each increase of 1 % in the dry matter there were increases in the consumption of silage dry matter of 0·45 and 0·59 lb/day on the high and low concentrate treatments respectively in 1957–58, and 0·62 and 0·66 lb/day in 1958–59. In both years changes in the intake of silage dry matter were accompanied by similar changes in the total daily intake of dry matter, and there was no tendency for the intake of hay to alter in a direction opposite to that of the silage.

Starch equivalent

The intake of starch equivalent was calculated from the dry-matter intake of each feed and the starch equivalent values given in Table 1. The difference in daily intake between the two feeding treatments showed minor variations during different weeks but in both years the animals on the high treatment consumed an average of 4 lb of starch equivalent per day more than those on the low treatment. The mean intakes are given in Table 3. The figures are only approximate because of the empirical nature of the values for the starch equivalent contents of the feeds, but they give an indication of the differences between the two treatments. The amount of starch equivalent consumed expressed as a percentage of Woodman's standards for the cow's requirement (Evans, 1960) averaged 122 and 103 % on the high and low treatments respectively.

Milk yield

The mean daily milk yields per cow for days 1–70, and 1–140, and the yields for the complete lactation are given in Table 4. There was very little difference between the mean yields of the animals on the high and low steaming-up treatments at any stage of lactation and, furthermore, there was no significant interaction between the feeding treatments before and after calving. The high treatment after calving resulted in higher yields than the low treatment after calving, but the differences

Table 4. *The mean values of milk yield and composition, and length of lactation*

	Treatment				Standard error of difference between means	Steaming-up means		Lactation feeding means	
	HH	HL	LH	LL		High	Low	High	Low
Milk yield									
Days 1–70, lb/day	44·1	38·8	42·8	40·8	±2·7	41·4	41·8	43·4	39·8
Days 1–140, lb/day	39·4	33·4	37·7	35·5	±2·4	36·4	36·6	38·6	34·4
Lactation,* lb	8820	7470	8370	7930	±650	8140	8150	8600	7700
Peak yield, lb/day	50·6	46·3	48·9	46·8	±3·3	48·4	47·8	49·8	46·6
Fat, %									
Days 1–140	4·18	4·38	4·33	4·19	±0·15	4·28	4·26	4·26	4·28
Lactation*	4·19	4·36	4·33	4·17	±0·13	4·28	4·25	4·26	4·26
S.N.F., %									
Days 1–140	8·78	8·67	8·85	8·62	±0·11	8·72	8·74	8·82	8·64
Lactation*	8·67	8·63	8·78	8·63	±0·12	8·65	8·70	8·72	8·63
Length of lactation, days	282	284	291	274	±9	283	282		279

* Lactation not exceeding 305 days.

were always slightly below the value required for a 5% level of significance. The coefficients of variation attached to the mean milk yields were high at 13% for both days 1–70 and days 1–140 and 16% for the complete lactation.

The mean lactation curves of the animals on the two feeding levels after calving are shown in Fig. 1. The effect of the steaming-up treatments on the shape of the curves was very small so mean curves of the HH and LH, and the HL and LL treatments have been given. The peak yield in week 3 on the low feeding level never reached that of the high level, and this difference was maintained with only minor variations until week 24 when the cattle first had access to pasture in the spring. After this time the curves were not so far apart, and in week 31 the difference in the

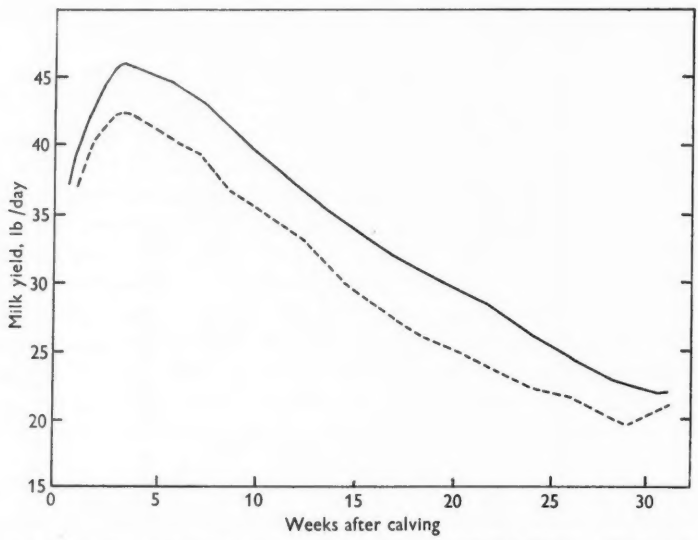

Fig. 1. The mean daily milk yields on the High (average of HH and LH) and the Low (average of HL and LL) concentrate treatments after calving. ——, High; – – – –, Low.

mean yield between the two groups was only about 1 lb/day because of a rise in the yield of the low concentrate group which was almost certainly the result of the good grazing.

Details of the peak yield, i.e. the mean daily value of the highest total production recorded in any period of 3 consecutive days, on the various treatments are given in Table 4. The differences between the mean values were not statistically significant, but again the error term was high and the coefficient of variation was 14%.

Milk composition

The mean fat and S.N.F. contents of the milk on each treatment for days 1–140 and for the whole lactation are shown in Table 4.

The differences in the composition of the milk as a result of the feeding treatments were in general small. The fat content on all treatments was greater than 4% and there were no statistically significant differences between them.

The steaming-up treatments did not appear to have any effect on the S.N.F. content of the milk, but in days 1–140 the high level of concentrate feeding after calving resulted in a mean value which was 0·18 % higher ($P < 0·05$) than the value obtained on the low level of feeding.

The mean S.N.F. values for each treatment in the two years of the experiment are shown in Fig. 2. In 1957–58, after the initial fall in the S.N.F. contents the values changed relatively little until the cows had access to pasture in late April 1958.

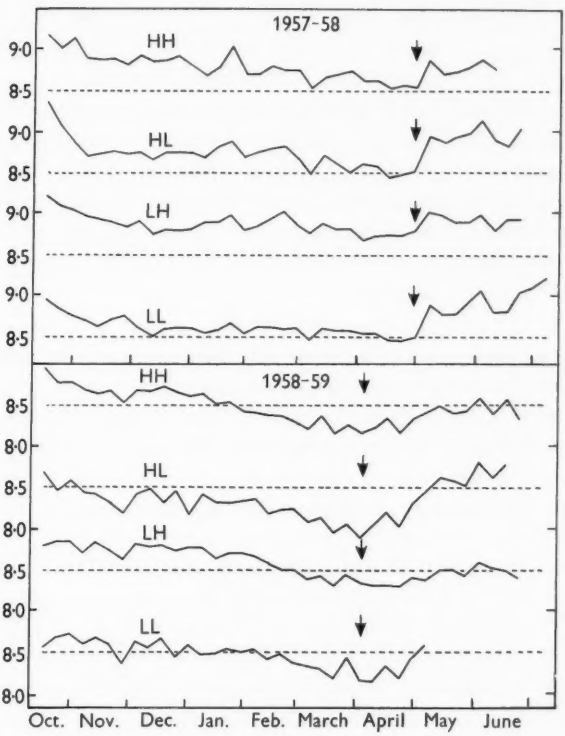

Fig. 2. The mean S.N.F. content (%) of the milk on the four treatments in 1957–58 and 1958–59. Each curve represents the mean value for four cows. The LL curve in 1958–59 is curtailed, because one cow was dried off. ↓, Indicates when grazing in the spring started.

The S.N.F. values then increased, and a comparison of the mean S.N.F. contents in the periods of 4 weeks before and 4 weeks after grazing started showed that the low concentrate group (mean HL and LL) increased by an average of 0·40 % and the high concentrate group (mean HH and LH) by 0·21 %. For 2 weeks immediately before the grazing season began, the S.N.F. values in the LL and the HL groups were below 8·5 %, whereas in the other two groups the mean values were never below this figure. In 1958–59 the S.N.F. values showed a distribution very different from that in the previous year. On the HL treatment the S.N.F. value fell to below 8·5 % in early November 1958, and did not increase until May 1959. On the other

three treatments the S.N.F. values were below 8·5% from January and February to May 1959, and in contrast to the previous year no large increase occurred immediately the cows started grazing in the spring.

Length of lactation

The maximum length of a lactation was fixed at 305 days and the mean number of days in the lactations on the various treatments is given in Table 4. The animals on the high level of concentrate feeding had on average 7 more days per lactation than those on the low treatment, but the difference was not statistically significant.

Response to extra feeding

From the milk yield and S.N.F. data for days 1–140 in Table 4 and the mean starch equivalent intakes in Table 3, it was calculated that the response to each additional 1 lb starch equivalent was 1·05 lb of milk and 0·045% S.N.F. The yield response agrees well with values found in other trials (Holmes *et al.* 1957; Castle *et al.* 1958; Broster *et al.* 1958) and the S.N.F. response is within the range of 0·04–0·06% recorded in short-term changeover trials (Holmes, Waite, MacLusky & Watson, 1956; Holmes *et al.* 1957).

Live weight

The live weights of the animals at three stages after calving are summarized in Table 5. Losses in live weight were recorded on all treatments in the first 10 weeks after calving with the smallest loss on the HH treatment. Between the tenth and twentieth week after calving the animals on the HH treatment completely regained

Table 5. *The mean live weights of the cows in the various groups* (*lb*)

Weeks after calving	Treatment				Lactation feeding means	
	HH	HL	LH	LL	High (HH and LH)	Low (HL and LL)
1	1072	1145	1120	1070	1096	1108
10	1055	1065	1058	994	1056	1030
20	1072	1077	1085	989	1078	1033

their lost live weight, the ones on the HL and LH treatments made a small gain and the animals on the LL treatment had a further small loss. On average the high treatment animals (mean HH and LH) decreased in live weight by only 18 lb in the 20 weeks after calving whereas the low treatment cows (mean LL and HL) decreased by 75 lb in the same period. After grazing began in the spring there were temporary falls in live weight probably due to changes in the contents of the digestive tract, particularly in the high group, but after this time there were gains in both groups until at the end of the lactation the mean weights were almost identical on both treatments.

The changes in live weight in the trial were broadly similar to the ones reported by Broster *et al.* (1958) from a similar feeding experiment.

DISCUSSION

Although the feeding treatments in this experiment were planned to provide very different intakes of concentrates, the differences in milk yield and composition on the four treatments were rarely significant even at the 5 % level (Table 4). Mean values often differed by 10 % or more, but the error terms were in general large, e.g. the coefficients of variation attached to the milk yield data varied from 13–16 % with eight replicates per treatment. These values compare unfavourably with values of 4–5 % obtained in relatively short changeover trials using the same herd and the same number of replicates (Holmes *et al.* 1957; Campling & Holmes, 1958; Castle *et al.* 1961). These changeover trials, however, were conducted during the declining phase of lactation after the peak yield had been passed, and this together with the shortness of the periods of treatment would help to reduce the variability of the results. An increase in the number of animals on the present experiment would have been expected to reduce the errors attached to the various measurements, but a very large increase would have been required to produce any appreciable reduction. In a similar type of continuous feeding trial with eighty-eight heifers (Broster *et al.* 1958) the coefficients of variation attached to the milk yield data were similar to those found in the present trial, and even having twenty-two replicates did not reduce the error term to as low a level as that in the changeover trials referred to above. As suggested by Lucas (1960) it seems that continuous trials should be used where the various treatments are such that nutrients may be stored and where effects of the different treatments may take some time to show, or where large carry-over effects are likely. Trials of the type reported in this paper can give valuable information on the effect of feeding treatments over a complete lactation, though they lack the precision obtainable in short changeover trials.

In this experiment the difference in the two levels of concentrate feeding before calving did not affect the yield and composition of the milk or the live weight changes of the cattle during the lactation that followed. A high level of feeding in late pregnancy is generally advised (Boutflour, 1943; Ministry of Agriculture, Fisheries & Food, 1960) but well-controlled studies of this feeding practice are relatively few and the results are somewhat conflicting (Broster, 1958). The steaming-up of cattle was found by Blaxter (1944) not to be as successful on a bulky roughage ration as on a ration of concentrates, but undoubtedly in our trial, with the good quality grazing available, the smaller amount of concentrates provided an adequate steaming-up ration. Ample pasture at the correct grazing stage was always available to the cattle and on this ration they approached calving in a fit and well-fed condition, but without excessive fatness, and no advantage appeared to result from feeding 3·3 cwt of concentrates per cow in the 5 weeks before calving instead of 1·6 cwt.

During lactation 25·8 cwt of concentrates per cow, or 4·12 lb per 10 lb of milk, were fed to the cows on the high post-calving treatment compared with 12·1 cwt per cow, or 2·19 lb per 10 lb milk, on the low post-calving treatment. The total lactation yield per cow averaged 860 gal on the high treatment compared with 770 gal on the low treatment, a difference of about 12 %, 1 lb of additional starch equivalent fed resulting in an additional 1·05 lb of milk. The cost of feeding the cows on the high post-calving treatment was £18·9 per cow greater than on the low post-calving

treatment, but since the value of the additional 90 gal of milk produced per cow was only £15·8 there was a deficit of just over £3 per cow for the high level of concentrate feeding during lactation compared with the low level. It is clear that of all the four treatments the low level of steaming-up followed by the low level of concentrate feeding during lactation gave the greatest profit per cow under the present method of valuing milk solely according to yield, but if payment on quality were to be introduced the estimated deficit of £3 per cow might well be considerably reduced since the S.N.F. content of the milk on the high post-calving treatment was 8·82% compared with 8·64% on the low post-calving treatment. The fat contents of the milk were on average unaffected by the different treatments.

The results obtained in this trial vary in certain aspects from those of Broster *et al.* (1958) but the differences can be largely explained by the different amounts of concentrates used in the two trials. Broster *et al.* obtained a response in milk yield to the higher level of steaming-up by comparing $\frac{1}{2}$ cwt and $2\frac{1}{4}$ cwt of concentrates per cow whereas in the present trial there was no response, but the amounts fed were considerably larger at 1·6 cwt and 3·3 cwt per cow. In addition, the leafy autumn herbage fed during the steaming-up period in our trial probably supplied more feed nutrients than the forage crops which Broster *et al.* used. After calving, the difference between the high and low rates of concentrate feeding per gallon were similar in both trials and the result showed that with adequate steaming-up the high rates were uneconomic in producing extra milk.

On average the milk in the 1958/59 period of the experiment contained 0·25% less S.N.F. than in 1957–58. This difference is attributed mainly to the different intakes of starch equivalent on the corresponding treatments in the two years (Table 3). The lower intake of starch equivalent in 1958–59 compared with that in 1957–58 was the result of both a reduction in the dry matter consumed and in the starch equivalent of the dry matter (Table 1). The lower quality of the feed produced in the summer of 1958 was thought to be due to the extremely wet and sunless weather. The abnormally bad weather in 1958 which affected the quality of home-grown feedingstuffs has been blamed for the low S.N.F. content of milk of herds over a wide area of England and Wales throughout the winter of 1958–59 (Report of the committee on milk composition in the United Kingdom, 1960; Joint committee for the control of milk quality, 1960).

An important example of the effect of feeding on milk composition is given by the seven cows which were used in this experiment in both years. Three of these cows happened to be on the high post-calving treatment in 1957–58 and on the low post-calving treatment in 1958–59, and the average S.N.F. content of their milk fell from 8·95% on the high treatment in the first year to 8·53% on the low treatment in the second year, while the average fat content increased from 4·02 to 4·44%. Although there would be a slight age effect, the decrease in the S.N.F. content would almost certainly be due to the lower intake of starch equivalent, while the higher fat content would result from the relatively greater roughage content of the feed (Rook, 1959). There were only four other cows which were used in both years. They happened to be on the low treatment in 1957–58 and on the high treatment in 1958–59. The average S.N.F. content of their milk was 8·62% in the first year and 8·72% in the second, the change from the low to the high concentrate treatment preventing any

decrease that might have occurred as a result of either an increase in age from one year to the next or the lower quality of the hay and silage in 1958–59. The average fat content of the milk of these four cows was 4·08% in 1957–58 and 4·15% in 1958–59.

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Enhancement of the flavour of Cheddar cheese by adding a strain of micrococcus to the milk

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Summary. Three strains of micrococci and two of pediococci were individually tested for effect on Cheddar cheese flavour by their addition to cheese milk. Only one of the micrococcal strains had a significant effect on flavour. In twenty-eight of the thirty-three trials with this strain the flavour of the experimental cheeses was appreciably better than that of the corresponding control cheeses; in the other five trials the experimental and control cheeses were similar in flavour.

This micrococcus did not grow appreciably during manufacture or ripening of the cheese, the peak number being present in the curd at the time of pressing. The count fell to less than 30% of the peak number after 1 week, and to less than 0·5% after 5 weeks.

The ways in which the micrococcus may have influenced the flavour of the cheese are discussed and attention is drawn to the possibility that bacterial action in the milk and the young cheese may play an important part in flavour development.

It has often been suggested that the characteristic flavour of cheese results to a large extent from the action of bacteria or fungi during the ripening process. In Cheddar cheese the starter streptococci (Perry, 1961) and the adventitious bacteria present in the milk or accidentally incorporated during manufacture may contribute to the final flavour. In the past it has often been assumed that only those strains of micro-organisms present in large numbers in mature cheese make any significant contribution to flavour. Hence, the classical approach to the problem of discovering the strains and species responsible for cheese flavour has been to inoculate cheese milk with organisms isolated from mature cheese of good flavour. In many of the early investigations various strains of lactobacilli were added (Lane & Hammer, 1935; Sherwood, 1939*a*, *b*), since this genus usually dominates the flora of normal mature Cheddar cheese. While inoculation of the milk with selected strains of lactobacilli has been shown to effect some improvement in flavour, numerous trials at this Institute (unpublished data) have not given any evidence that lactobacilli themselves are responsible for the characteristic Cheddar flavour. More recently attention has been directed to genera which form only a small proportion of the microflora at maturity. Thus propionibacteria (Harris & Hammer, 1940), leuconostocs (Tittsler, Sanders, Lochry & Sager, 1948), enterococci (Tittsler *et al.* 1948; Freeman, 1959) and micrococci (Harris & Hammer, 1940; Alford & Frazier, 1950) have been added to the vat during cheesemaking in attempts to improve flavour in Cheddar cheese.

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In 1954 some samples of raw milk which happened to remain undisturbed in this laboratory for several weeks were observed to possess a distinctly cheesy aroma. From these samples three micrococci (L_1, L_9 and M_{10}) and a pediococcus (G_2) were isolated on nile-blue-dyed butterfat agar (Stark & Scheib, 1936). A number of trials were subsequently made with these four strains and a pediococcus (A_2, isolated by Dacre, 1958) to ascertain their influence on the flavour of Cheddar cheese. The most promising of these strains (micrococcus L_1) was more exhaustively tested for influence on Cheddar flavour at intervals during the 1959–60 dairy season.

EXPERIMENTAL

Method of manufacture

The cheeses were made following the standard method of manufacture which has already been described (Perry, 1961). The milk taken from the bulked supply of five herds in which the Jersey breed predominated, was always of good bacteriological quality. It was flash-pasteurized at 150 °F, then divided into 80-gal batches in the required number of 80-gal vats. The control cheese was made on the preceding day or, more often, the cheesemaking schedule was arranged so that the control vat was rennetted before the inoculation of the experimental vat(s) with the test culture. These procedures, combined with other obvious precautions, reduced the risk of accidental cross-contamination of the control vat with the test organism.

Table 1. *Strains of starter streptococci and of added micrococci used in making forty-five experimental cheeses*

Strain	No. of experimental cheeses	Strains of starter streptococci
	(*a*) Preliminary experiments	
Pediococcus A_2	2	HP (1)*; ML_3 (1)
Pediococcus G_2	2	KH + R_1 (2)
Micrococcus L_1	5	KH + R_1 (4); ML_1 + ML_3 (1)
Micrococcus L_9	5	HP (1); ML_3 (1); KH + R_1 (2); C_{13} + E_8 (1)
Micrococcus M_{10}	3	HP (1); ML_3 (1); HP + US_3 (1)
	(*b*) Extended investigation	
Micrococcus L_1	28	HP(5); ML_3 (4); ML_2 (2); ML_8 (2); C_{13} (1); KH (1); BR_4 + LT_8 (2); C_{13} + E_8 (2); E_8 + ML_4 (2); FC_4 + R_1 (2); TR + Z_8 (2); HP + US_3 (1); KH + R_1 (1); ML_1 + ML_4 (1).

* Figures in parentheses indicate the number of times the various starters were used.

In all, three strains of *Streptococcus lactis* and twelve of *Str. cremoris* were used as cheese starters either singly or in pairs as is shown in Table 1. Details of the physiological and biochemical characteristics of the five strains of micrococci tested for influence on cheese flavour are shown in Table 2. Shaw, Stitt & Cowan (1951) have proposed that micrococci should be included in the genus *Staphylococcus*. However, in this paper the term 'micrococcus' is used to describe strains L_1, L_9 and M_{10}. The test cultures were grown in yeast milk (YM) at 30 °C for 4 days. Inocula of

0·01, 0·1 or 1% (usually 0·1%) of YM cultures were added to the vat before the addition of the starter. An equal volume of uninoculated YM was added to the control vat.

Table 2. *Biochemical and physiological characteristics of the five strains*

	Pediococci		Micrococci		
	A_2	G_2	L_1	L_9	M_{10}
Catalase	−	+	+++	+++	+++
Terminal pH (GLYPB)*	4·1	4·2	4·6	4·6	4·7
Coagulase (tube)	.	.	−	−	−
Growth, NaCl 6%	+	+	+	+	+
8%	−	−	+	+	+
10%	−	−	+	+	+
12%	−	−	+	+	+
Gelatin liquefied	−	−	+	+	+
Milk agar digestion	−	−	−	−	+++
NH_3 from arginine	+	+	+	+	+
Haemolysis (calf blood)	.	.	−	−	−
Growth broth pH 9·0 (48 h)	−	−	+	+	+
Growth nutrient agar	+	+	+++	+++	+++
Aesculin hydrolysis	+	+	+	+	+
Voges-Proskauer reaction	.	.	+++	+++	++
Lipolysis, tributyrin agar	.	.	+++	+++	+++
butterfat agar	.	.	++	++	++
Fermentation:					
Arabinose	+	+	.	.	.
Dextrin	+	+	.	.	.
Fructose	+	+	.	.	.
Galactose	+	+	.	.	.
Glucose	+	+	+	+	+
Glycerol	+	.	±	+	+
Lactose	+	+	+	+	+
Maltose	+	+	.	.	.
Mannitol	−	−	−	−	+
Melezitose	−	−	.	.	.
Melibiose	−	−	.	.	.
Raffinose	−	−	.	.	.
Salicin	+	+	−	−	−
Sorbitol	−	.	.	.	.
Sucrose	−	.	.	.	.
Trehalose	−	+	.	.	.
Xylose	−	−	−	−	−

+, ++, +++ = Positive reactions; ± = weak positive reaction; − = negative reaction; . = not tested.

All strains were Gram positive cocci arranged in pairs and tetrads; the micrococci often showed loose clusters. The fermentation reactions of the pediococci were determined in the medium of Wheater (1955) and of the micrococci in peptone water.

* GLYPB is glucose lactose yeast phosphate broth (Robertson, 1960*b*).

Bacteriological examinations

To check the absence of gross bacteriological abnormalities, starter and non-starter counts of many of the cheeses were made using the methods described by Robertson (1960*a*) and Perry (1961). These determinations were made on samples of the milk in the vat before and after adding the experimental cultures and following the addition of the starter, also on the cheese at dressing and at 1, 3, 5, 9, 14 and 26 weeks. Six of the cheeses to which L_1 had been added were examined in greater detail,

fifty non-starter isolates being picked from appropriate plates at each sampling. As no micrococci were detected in the milk used or the control cheeses, it was assumed that the micrococci detected in the cheeses made from milk to which strain L_1 had been added were in fact L_1.

RESULTS

Composition of the cheeses

Details of the manufacturing times and acidities for both control and experimental cheeses are summarized in Table 3, and in Table 4 the compositional characteristics of the cheeses are given. The cheeses were all within the normal compositional range of New Zealand cheese. As a result of the careful regulation of cheesemaking operations the principal objective of producing closely similar values for pH, water in the non-fat substance (W.N.F.S.) and salt in moisture concentrations in corresponding control and experimental vats was in fact achieved.

Table 3. *Manufacturing data*

(80 gal of flash-pasteurized milk; 1½% of single strain starter; 80 ml of rennet)

	Control cheeses (40)*		Experimental cheeses (45)		Differences within pairs of cheeses†	
	Average	Range	Average	Range	Average	Range
% acidity at rennetting	0·16	0·15–0·18	0·16	0·15–0·17	0·00	−0·01–0
% acidity at cutting	0·10	0·09–0·11	0·10	0·09–0·11	0·00	−0·01–0
% acidity at running	0·14	0·13–0·15	0·14	0·13–0·15	0·00	−0·01–+0·01
% acidity at drying	0·15	0·14–0·17	0·15	0·14–0·17	0·00	0–+0·01
% acidity at milling	0·48	0·35–0·58	0·48	0·35–0·56	−0·01	−0·05–+0·01
% acidity at salting	0·70	0·60–0·80	0·71	0·59–0·80	+0·04	−0·04–+0·05
Time, rennetting to drying (h and min)	2.35	2.20–3.05	2.36	2.20–3.00	0.00	−0.20–+0.15
Time, drying to salting (h and min)	2.29	2.00–3.10	2.29	2.00–3.00	0.00	−0.30–+0.35
Total making time (h and min)	5.04	4.40–6.05	5.04	4.40–6.05	0.00	−0.50–+0.50

* Some control cheeses common to more than one experimental cheese.

† + indicates values for experimental cheese (L_1) greater than for control cheese of the same pair.

Flavour of the cheeses

The flavours of the cheeses were assessed when they were a fortnight old and at monthly intervals thereafter by the same four or five members of an experienced cheese tasting panel. The members of the panel worked together but did not know the identity of the cheeses they were examining. A summary of the results of the preliminary experiments is shown in Table 5. The improvement of cheese flavour which resulted from the addition of strains A_2, G_2, L_9 and M_{10} to cheese milk was less than from the addition of L_1. Cheeses made using strain L_1 were consistently of fuller flavour than the controls, sometimes when only a fortnight old.

The results of more extensive investigation of the influence of L_1 are summarized in Table 6. In no instance was the flavour of an experimental cheese inferior to that of its control but the results based on flavour at maturity were divisible into two groups. In the first, and by far the larger group (twenty-eight of thirty-three com-

parisons), the experimental cheeses were of better flavour than their controls, while in the second group (five of thirty-three comparisons) there were no detectable flavour differences. All the control cheeses in the first group were of good quality; their flavours showed the slight differences normally encountered in a group of mature Cheddar cheeses. Of the five comparisons in which no flavour difference was detected three of the control cheeses were of exceptionally good Cheddar flavour,

Table 4. *Analysis of cheeses at* 14 *days*

(80 gal flash-pasteurized milk; 1½% single strain starter; 80 ml rennet)

	Control cheeses (40)*		Experimental cheeses (45)		Differences within pairs of cheeses†	
Analysis at 14 days	Average	Range	Average	Range	Average	Range
Moisture, %	34·5	32·8–36·2	34·5	33·2–35·7	−0·2	−0·9–+0·3
Fat, %	37·2	35·5–38·8	37·2	35·9–38·5	+0·2	−0·1–+0·5
F.W.F.S., %	56·6	55·4–57·9	56·7	55·4–58·4	+0·2	0–+0·9
W.N.F.S., %	54·8	52·0–56·8	54·8	53·3–56·7	−0·3	−1·0–+0·2
Salt, %	1·6	1·3–2·0	1·6	1·3–2·0	+0·1	−0·1–+0·4
Salt in moisture, %	4·6	3·4–5·7	4·6	3·6–5·7	+0·2	−0·2–+0·4
pH	5·0	4·9–5·2	5·0	4·9–5·2	−0·1	−0·1–0

* Some control cheeses common to more than one experimental cheese.
† + indicates values for experimental cheese (L_1) greater than for control cheese of the same pair.

Table 5. *Flavours of seventeen cheeses made during preliminary experiments*

	Degree of improvement* in flavour as compared with control cheese		
Strain	2 weeks	8 weeks	26 weeks
Pediococcus A_2			
Experiment 1	0	0	+++
Experiment 2	0	0	0
Pediococcus G_2			
Experiment 1	0	0	+++
Experiment 2	0	0	0
Micrococcus L_1			
Experiment 1	++	+++	++++
Experiment 2	+	++	++++
Experiment 3	+	+++	++++
Experiment 4	0	0	++++
Experiment 5	+	++	+++
Micrococcus L_9			
Experiment 1	0	0	+++
Experiment 2	0	0	+++
Experiment 3	0	0	0
Experiment 4	0	0	0
Experiment 5	0	0	0
Micrococcus M_{10}			
Experiment 1	0	0	++
Experiment 2	+	++	0
Experiment 3	+	0	0

* 0, no improvement; + to ++++, increasing improvement (++ was considered to be a worthwhile improvement in flavour).

leaving little room for improvement, while the other two had undesirable flavours. It appears, therefore, that the beneficial effect of L_1 was apparent only when the control cheeses were of good flavour. No effect was apparent when the flavour of the control cheese was exceptionally good nor did L_1 prevent the development of some undesirable flavours.

Table 6. *Summary of flavour examinations of thirty-three L_1 cheeses*

Type of starter	No. of experimental cheeses	Degree of improvement* in flavour as compared with control cheese		
		2 weeks	8 weeks	26 weeks
Str. lactis (one strain)	8	++ (2) + (1) 0 (5)	+++ (5) ++ (2) 0 (1)	++++ (6) +++ (2)
Str. lactis + *Str. cremoris* (one strain of each)	1	+ (1)	++ (1)	+++ (1)
Str. cremoris (one strain)	7	++ (3) 0 (4)	+++ (5) 0 (2)	++++ (5) 0 (2)
Str. cremoris (two strains)	17	++ (1) + (2) 0 (14)	+++ (10) ++ (1) 0 (6)	++++ (12) +++ (2) 0 (3)
Total	33	++ (6) + (4) 0 (23)	+++ (20) ++ (4) 0 (9)	++++ (23) +++ (5) 0 (5)

Values in parentheses indicate the number of cheeses in each category.
* 0, no improvement; + to ++++, increasing improvement.

When 2 weeks old, a slight beneficial effect from the addition of L_1 was noted in ten of the twenty-eight cheeses which subsequently showed an effect. In the experiments where the effect of L_1 was apparent at 2 weeks the flavours of the control cheeses were somewhat abnormal, being slightly sharp or slightly bitter. These 'off' flavours disappeared as ripening proceeded but the superior flavour of the L_1 cheeses persisted.

At maturity a characteristic 'lactis' flavour (Perry, 1961) was detected in all of the cheeses made using *Str. lactis* strains, including those made with a mixed starter of *Str. lactis* and *Str. cremoris*. When first examined after 2 weeks' ripening almost all of the lactis control cheeses possessed the slightly 'dirty', slightly 'fruity' off-flavour; at this age a 'lactis' flavour similar to that of the controls was present in five of the nine L_1 experimental cheeses. Presumably the development of 'lactis' flavour was prevented or masked by the L_1 in the other four experimental cheeses. As ripening proceeded the 'lactis' flavour increased in intensity and became apparent in all the experimental cheeses made with *Str. lactis* but remained less pronounced than in the control cheeses (Table 6).

The results obtained with lactis cheeses may be contrasted with those found for cremoris cheeses. In the latter, L_1 had no detectable effect on the flavour of five of the twenty-four experimental cheeses even at maturity (see above).

The size of the L_1 inoculum appeared to have little effect on the degree of flavour enhancement. In two sets of trials comparisons of inocula of 0·01 with 0·1 % and

of 0·01 with 1 % were carried out. In the former trials the experimental cheeses containing 0·1 % inocula appeared to have a slightly stronger Cheddar flavour after 2 months' ripening, this difference not being observed when the cheeses were older. Against this, however, no differences in flavour were noted in the experiments with 0·01 and 1 % inocula at any age.

Bacterial flora and bacteriophage

The total starter and non-starter counts and the numbers of phage particles found were similar to those reported in other investigations (Robertson, 1960; Perry, 1961). In agreement with the findings of Dawson & Feagan (1957) and Perry (1961) the *Str. lactis* strains reached higher numbers in the curd and survived longer in the cheese than *Str. cremoris* strains.

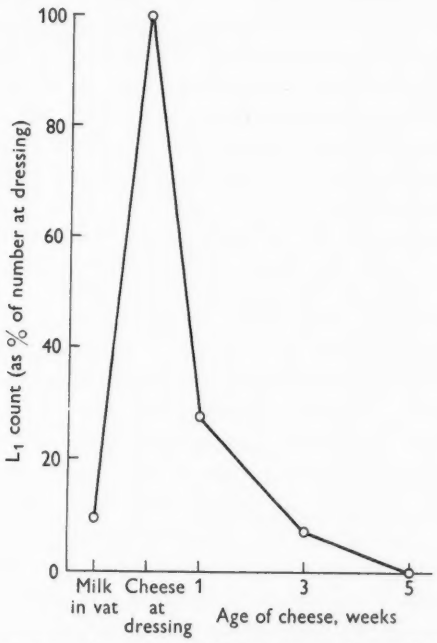

Fig. 1. Mean numbers of survivors of strain L_1 in six cheeses (0·1 % inoculum, 10^9 organisms/ml).

More detailed bacteriological examinations were made of six of the experimental cheeses (Fig. 1). In these L_1 comprised 100 % of the non-starter flora for the first 3 weeks of the ripening period. Contrary to the finding of Alford & Frazier (1950) with other micrococci, strain L_1 did not multiply appreciably during or after cheese-making. The approximately eight-fold increase in numbers of L_1 organisms/g from the milk in the vat to the cheese curd at dressing (Fig. 1) was probably due to retention of cells in the curd as moisture was expelled during manufacture. The numbers of L_1 surviving in the cheeses fell rapidly to less than 30 % of those at dressing after 1 week, and to less than 0·5 % after 5 weeks. At 9, 14, and 26 weeks no strains morphologically similar to L_1 were isolated (Fig. 1).

DISCUSSION

The cheeses reported upon in this paper were of good quality, were manufactured under standard conditions (Table 3) and the control and experimental cheeses were of similar chemical composition and pH (Table 4). The series comprised forty-five experiments of which thirty-three concerned strain L_1. The trend in flavour was always in the same direction in the experiments which involved sixteen strains of starter streptococci and three levels of L_1 inoculum, so that the beneficial effect on final cheese flavour of adding a culture of L_1 to the milk in the vat may be considered well established. The way in which L_1 acts is uncertain.

It is clear that, under the conditions used, L_1 does not proliferate in the cheese. The flavour-enhancing substances or flavour precursors may have been: (i) introduced into the cheese as preformed entities in the inoculum; (ii) produced within the cheese as a direct result of the presence of enzymes of strain L_1; or (iii) due to a modification of the flora as a result of the presence of L_1. If it is supposed that the enzymes of L_1 are responsible for the improved flavour, and that the enzymes are extracellular, then they would have been present in the culture added and more could have been produced by the non-proliferating but still viable L_1 cells. If they were intracellular they would have been liberated following the death and subsequent autolysis of the cells. On the evidence available it is not possible to decide which of the three possibilities is most likely although the second alternative is preferred as this offers the simplest explanation of why the flavour differences were not great at 2 weeks but were well defined by 2 months, changing little thereafter.

The absence of any definite effect on flavour development of a hundred-fold change in the level of L_1 addition is difficult to explain. One possibility is that the quantity of flavour-precursors is limited or becomes available only slowly in cheese. If this were so, 10^5 L_1 organisms/g (0·01 % inoculum) may be almost as effective as 10^7 L_1 organisms/g in liberating the flavour-enhancing substances.

Recent work (Kristoffersen & Gould, 1960; Walker, 1961) has indicated the possible connexion of hydrogen sulphide and volatile fatty acids with cheese flavour. It is of interest, therefore, that of the five strains tested only strain L_1 liberated H_2S from suitable media (Robertson, unpublished data). This strain is also strongly lipolytic. Since micrococci are regularly found in raw milk, an intensive effort to isolate further strains capable of improving cheese flavour would appear worthwhile.

The results presented in this paper clearly demonstrate the fallacy of assuming that it is necessarily the organisms present in mature cheese which are responsible for Cheddar flavour. Strain L_1 was not only unable to proliferate in cheese but rapidly decreased in numerical importance. Many other species which thrive in milk and on dairy utensils find the conditions in cheese unsuitable. This is particularly true of the species which are unable to tolerate high salt or acid concentrations and anaerobic conditions. We consider that such species, particularly those with lipolytic and proteolytic activity, may play a part in flavour development.

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A laboratory method for the investigation of milk deposits on heat exchange surfaces

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Summary. An apparatus and procedure for the laboratory investigation of some of the factors which control milk deposits on heat exchange surfaces is described. The use of the method has been illustrated by applying it to determine how the amount of deposit varies with the acidity of the heated milk.

The deposits which form on heat exchange surfaces when milk is heated are of considerable interest to the dairy industry for two main reasons. First, their removal is an important and difficult part of cleaning operations, and secondly the length of a processing run may be limited by restriction of the flow passages through the build-up of deposits. The use of processing temperatures above about 85 °C, which has become more common in recent years, has aggravated these difficulties.

Comparatively few studies have been made of the subject. Those which have been in any way quantitative have relied on passing relatively large quantities of milk through small-scale heat exchangers, and measuring the rate of build-up of deposits by increase in the pressure required for flow (Bell & Sanders, 1944), or by determining the deposits after an experiment visually (Leeder & Martin, 1952) or by weighing (Gynning, Thomé & Samuelsson, 1958). Thus Bell & Sanders showed that the rate of deposition could be reduced considerably by preheating the milk, and this method has been used widely in industry. Leeder & Martin reported a reduction in deposits by the use of an electrical device, the 'Aquastat'. Gynning *et al.* showed that both increased acidity and increased air content of milk increased the amount of deposit. They showed also that certain operating conditions, such as high temperature differentials between milk and heating medium, and low milk velocities, encouraged the formation of deposits.

In any study of the chemical properties of milk, and of the surface conditions, which influence the amount of deposit on a heating surface, it is clearly an advantage to have a method of investigation requiring small volumes of milk and suitable for repeated experiments from a common bulk. In this laboratory a method has been evolved suitable for investigating the effects on rate of deposition of a limited range of variables, such as chemical composition, heating surface conditions and some pretreatment processes which do not affect the air content of the milk.

It was found that by using a temperature differential of about 15 deg C and with an air content typical of that for bulk milk (about 30 ml/l.), adequate amounts of

deposit could be obtained from small quantities of milk with a short heating time. Thus repeated experiments on a common bulk of milk could be made.

Deposits were formed from separated milk on an oil-heated stainless steel tube, under controlled conditions of milk temperature, heating temperature, duration of heating, and aeration of the milk as determined by the pretreatment processes. The deposits were removed by caustic soda solution and estimated by the biuret reaction.

DESCRIPTION OF APPARATUS

A partly sectional drawing of the apparatus is shown in Fig. 1. Milk is contained in the stainless steel vessel which is elliptical in section and thermally insulated with felt. The heating surface is a stainless steel U-tube, 0·25 in. internal diameter and

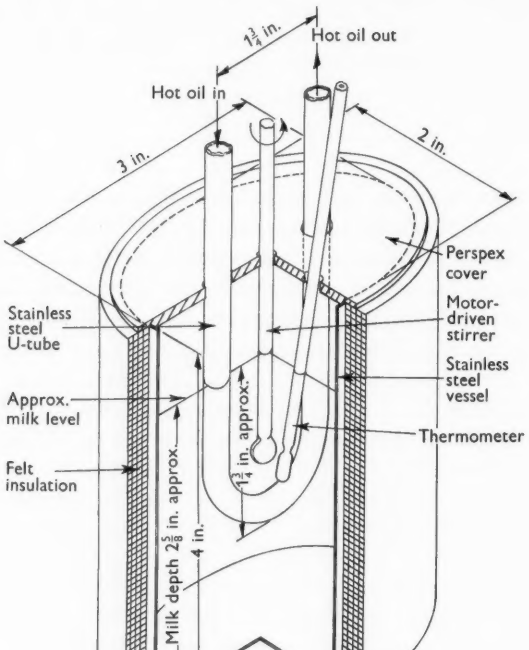

Fig. 1. Isometric sketch of apparatus.

0·30 in. external diameter, fitted with a threaded brass union at each end. The U-tube is connected by the unions to lagged pipes through which oil can be pumped by a constant-flowrate pump from a thermostatically-controlled oil bath.

In operation, the stainless steel vessel is supported around the U-tube so that the tube is immersed in the milk. The vessel has a Perspex cover to prevent excessive evaporation from the milk surface. Small holes in the cover give entry for the heating coil, a motor-driven stirrer and a thermometer; the latter are immersed in the milk near to the heating coil.

METHOD OF OPERATION

Formation of deposits

A 200 ml quantity of separated milk is used for each test. The milk is preheated in a flask or beaker immersed in an oil bath at 100 °C and is stirred vigorously until a temperature of 80–85 °C is reached. The U-tube is preheated by circulating hot oil at 100 °C for a few seconds, and the vessel is preheated with hot water. The milk is then transferred to the vessel from the flask or beaker, the vessel placed in position around the U-tube, and the oil pump started. The stirrer is operated at a speed to give good stirring without causing frothing. With a heating oil temperature of 100 °C, the milk temperature measured by the thermometer remains steady at about 85 °C.

After a 30-min period the oil pump and stirrer are stopped and the vessel removed from the U-tube. The U-tube is rinsed with distilled water and any free milk skin, as distinct from deposit on the tube, is removed by hand.

The deposit on the tube consists, with normal milk, of clearly visible nodules covering most of the surface of the tube, but possibly with clear areas between them. With milk acidified to about pH 6·2, the deposit increases to become a white, spongy deposit up to 0·05 in. thick, covering the whole of the immersed part of the tube.

Removal of deposits

The stainless steel vessel is rinsed with water, and filled with 220 ml of 5% (w/v) NaOH solution (analytical grade) at room temperature. It is replaced around the U-tube and the stirrer is operated at high speed. For deposits from normal milk, the oil pump is operated on a 50% time cycle, 5 min on and 5 min off, to raise the temperature to 40–50 °C and so improve the dispersion of the deposits. With the thick deposits from acidified milk it is better to avoid heating until most of the deposits have been removed, either by dissolving or physical action, otherwise discoloration of the protein solution may occur. A 30-min cleaning period is generally sufficient, although up to 40 min may be needed. The solution is then poured into a beaker and allowed to cool.

The U-tube is prepared for a further run by rinsing with distilled water and wiping with damp cotton wool, and the Perspex cover is also wiped with cotton wool.

The deposit consists of protein and mineral matter. Both are removed by the NaOH solution, but some of the mineral matter remains in suspension. The insoluble material may be removed by centrifugation, but it is convenient merely to allow the solution to stand for a few hours to obtain a clear solution for protein determination.

Estimation of deposits

A method based on the biuret reaction was selected for determining the amount of protein in the solution made from the deposit. This reaction involves the formation in alkaline solution of a purple complex between copper salts and substances containing two or more peptide bonds. It is particularly convenient since it can detect low protein concentrations, and requires alkaline conditions. The alkaline conditions already exist as a result of the cleaning method adopted.

The method is based on that given by Gornall, Bardawill & David (1949), slightly

modified to improve its sensitivity. Ten ml quantities of each of the protein solutions are measured into test tubes, and to each is added 1 ml of the biuret reagent. The tubes are inverted once to mix the reagent and solution, and are then allowed to stand for at least 30 min for the purple colour to develop. The optical densities are then measured relative to a blank of NaOH solution with added reagent, at a wavelength of 550 mμ. For the Unicam SP 600 spectrophotometer used in this work, 1 or 2 cm parallel-sided optical cells were found suitable. Two separate 10 ml quantities of each protein solution were taken and each optical density measured twice. The mean of the four readings was taken as the optical density for that protein solution.

The biuret reagent consists of 0·30 g of copper sulphate ($CuSO_4.5H_2O$) dissolved

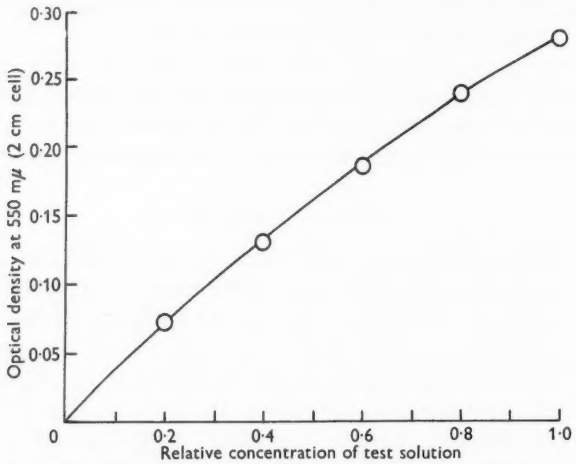

Fig. 2. Optical density–concentration relationship for modified biuret test.

in 50 ml of distilled water, to which is added 0·60 g of sodium potassium tartrate. The reagent must be made up freshly and used within 24 h. The tartrate is needed to stabilize the copper sulphate in the presence of caustic soda (Gornall *et al.* 1949). With lower concentrations of tartrate a cloudiness developed in the control NaOH solution when the reagent was added. With higher concentrations the tartrate was found to inhibit the colour formation to an undesirable extent.

A solution was prepared by dissolving the deposit from milk acidified to about pH 6·2 in 5% NaOH solution. By the addition of further 5% NaOH, more dilute solutions having concentrations of 0·2, 0·4, 0·6 and 0·8 times the original concentration were prepared. The relation between concentration and the optical density in the resulting biuret solution is shown in Fig. 2. It can be seen that the optical density increased almost linearly with increasing protein concentration, but with a slight progressive decrease in sensitivity.

Applicability of the biuret measurement

The biuret reaction involves the estimation of protein in solution. However, the deposit which is being measured certainly consists both of protein which passes into solution and of mineral matter which does not, and also of mineral matter which dissolves in the NaOH solution during cleaning. The biuret measurement is a satisfactory indication of the total deposit only if the mineral and protein components are sufficiently closely related over the range of conditions for which the method is used to investigate deposition.

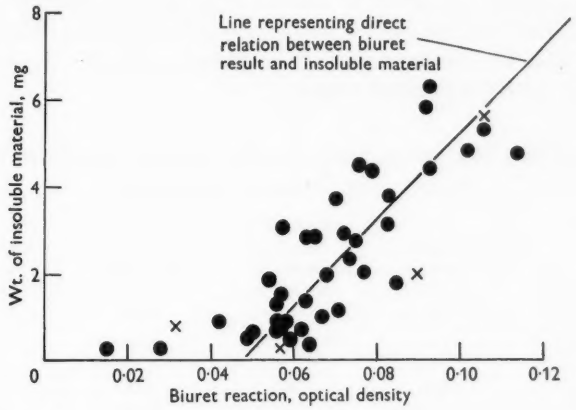

Fig. 3. Relation between protein content as measured by biuret reaction and weight of insoluble material. ×, Points used in Table 1.

Individual samples of deposit were prepared showing a range of amounts of deposition, the variation either representing the difference between individual milk samples or being caused deliberately by change in pretreatment which varied the air content of the milk. In addition to the spectrophotometric measurement, the insoluble material in the NaOH after cleaning was removed by vacuum-assisted filtration through sintered-glass Gooch crucibles (porosity grade 3) and was measured by weighing after rinsing the crucibles and drying them at a temperature of 110 °C for 1 h. The relation between mass of insoluble material and optical density of the filtrate in the biuret measurement is shown in Fig. 3. When the optical density was higher than about 0·05, the insoluble material varied directly with the optical density. Below this optical density there was no insoluble material.

This shape of curve can be simply explained on the assumption that the result of the biuret reaction is linearly related to the total amount of mineral matter, but that the 220 ml of 5% NaOH cleaning solution is able to hold about 5 mg of mineral matter in solution. This latter figure is obtained by extrapolation of the linear part of the curve, above 0·05 optical density, back to zero optical density.

It follows that the result of the biuret reaction is linearly related to the sum of the protein and mineral components, i.e. the total amount of deposit, but the scatter of the points on the linear part of the curve remains to be explained. This scatter is

probably caused by variations in the amount of dissolved mineral matter between the different experiments. The dissolved matter appears to depend on a number of factors: for example a clear filtrate which is left to stand will invariably deposit a further amount of insoluble material. Four experimental solutions were prepared which gave widely different points in the distribution shown in Fig. 3 (points indicated by a cross). For each solution, the dissolved mineral matter was estimated by extrapolation to zero optical density of a line at 45° through the corresponding point relating insoluble mineral matter to optical density. On the assumption that the amount of dissolved material may be estimated by a measure of the phosphorus content of the filtrate, probably existing as phosphate, the phosphorus content was determined. The results are given in Table 1. The ratio of phosphorus content to estimated total dissolved deposit is much more constant than the other variables, which suggests that the dissolved part of the deposits varied between samples in the way represented by the estimated values.

Table 1. *Relation between estimated dissolved mineral deposit and phosphorus content of the filtrate for four samples*

Optical density	Insoluble mineral deposit, mg	Estimated dissolved mineral deposit (*a*), mg	P content in solution (*b*), mg	P/estimated dissolved deposit (*b*/*a*)
0·090	1·96	7·0	0·35	0·050
0·057	0·28	5·5	0·28	0·049
0·106	5·61	5·0	0·31	0·062
0·032	0·80	2·4	0·15	0·062

It seems therefore that the biuret reaction is measuring something closely related to the total quantity of deposits, and that the scatter in the insoluble matter–optical density curve is related not to the biuret reaction, but to variations in the proportion of the total mineral matter (as represented by phosphorus-containing compounds) which is insoluble in the 5 % NaOH solution.

ASSESSMENT OF EXPERIMENTAL VARIABLES

Time of heating

Although a heating time of 30 min was used from the early stages of development of the method, there was no indication that this was in fact the optimum time. A series of tests was therefore made using the same bulk of separated milk and a constant heating oil temperature of 100 °C, with heating times of 7½, 15, 30 and 60 min. The variation in deposits, as given by the variation in optical density measured with a 1 cm optical cell, is shown in Fig. 4. It can be seen that the standard heating time of 30 min gave a reasonable compromise between the amount of deposit and the duration of heating. Longer heating times did not give a corresponding increase in the amount of deposit.

Temperature of heating

To determine the effect of variation in the temperature of the heating oil, a series of tests was made using the same bulk of separated milk with a constant heating time of 30 min and oil temperatures of 96, 100, 105 and 110 °C. The final temperature reached by the milk during heating rose from 83·5 °C at the lowest oil temperature to 86·5 °C at the highest. The variation of optical density (for a 1 cm optical cell) with oil temperature is given in Table 2.

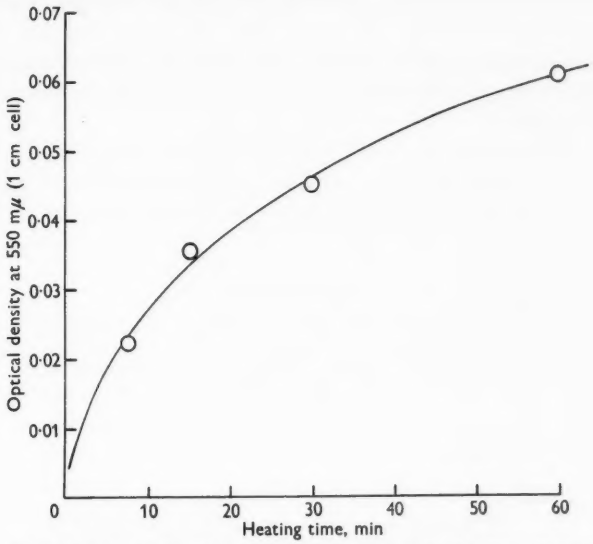

Fig. 4. Effect of variation in heating time on quantity of deposits (heating oil temperature 100 °C).

Table 2. *Variation of deposits as measured by optical density, for a constant heating time of* 30 *min and different heating oil temperatures*

Oil temperature, °C	Optical density for 1 cm cell
96·0	0·026
100·0	0·059
105·0	0·057
110·0	Discoloration prevented measurement

With the highest temperature, the deposit was burnt on to the tube very firmly and had a golden yellow colour, presumably caused by the Maillard reaction. This yellow colour persisted in the protein solution after clearing, and interfered with the measurement of the colour from the biuret reaction.

There was a slight indication of this yellow colour in the sample heated with oil at 105 °C, but this did not interfere with the reaction, as shown by a full spectrophotometric comparison of the colour curves given by the biuret reaction. However, this temperature gave no increase in deposits over an oil temperature of 100 °C, so that the latter seemed to be the most suitable value.

Reproducibility of results

Experience showed that the first experiment on any one day often gave low results. It is thought that this may have been caused by the condition of the heating surface, so this first result is excluded in considering the reproducibility of the results.

To determine reproducibility, a series of experiments was made with a common bulk of separated milk using the standard preheating, heating and measuring procedures. Three experiments were made on the first day and four on the following day after overnight storage of the milk in a refrigerator. The results are collected in Table 3.

It can be seen that repeatable results were obtained. There seems to be a slight indication of an increase in deposits on the second day, but the increase is not large enough to be conclusive.

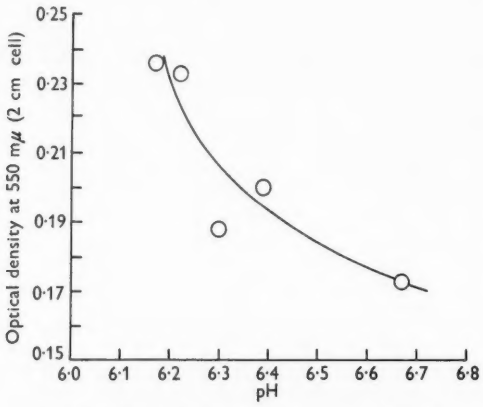

Fig. 5. Effect of milk pH on amount of deposits.

Table 3. *Reproducibility of results for one bulk of milk, using the standard preheating, heating and measuring procedures with a 2 cm optical cell*

Day no.	Experiment no.	Sample	Duplicate optical density readings	Mean
1	1	*a*	0·134, 0·135	0·133
		b	0·132, 0·131	
	2	*a*	0·142, 0·144	0·141
		b	0·138, 0·139	
	3	*a*	0·137, 0·137	0·136
		b	0·137, 0·133	
2	1	*a*	0·138, 0·135	0·142
		b	0·148, 0·146	
	2	*a*	0·149, 0·145	0·149
		b	0·151, 0·151	
	3	*a*	0·140, 0·142	0·146
		b	0·152, 0·150	
	4	*a*	0·137, 0·140	0·140
		b	0·141, 0·141	

PRELIMINARY EXPERIMENTAL RESULTS

As a preliminary application of this method, the effect of pH was examined. A bulk of raw, separated milk was divided into five parts. One of these was left at its original pH of 6·67. The others were acidified with 0·1N-HCl, to give different pH values down to pH 6·17. The milks were used for the formation and estimation of deposits by the standard procedure. A further milk sample acidified to pH 5·99 clotted in the heating vessel.

The variation with pH of the amount of deposit, as given by the optical density, is shown in Fig. 5. The results agreed with common experience, in that the amount of deposit increased with falling pH, slowly at first but then more rapidly as complete coagulation by heat was approached.

I acknowledge the assistance of Mr L. D. R. Turner in constructing the apparatus and in performing many of the experiments. I thank also Dr R. L. J. Lyster for his advice, and Dr R. Dawson for his help with the measurements.

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Estimation of oestrogenic activity in red clover (*Trifolium pratense*) using ovariectomized immature mice

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Summary. Two experiments concerned with the testing of plant material for oestrogenic activity, utilizing vaginal reactions and uterine weight changes in immature ovariectomized mice are reported.

In the first experiment the effects of varying the feeding period of an oestrogenic diet, containing 20 % of freeze-dried red clover, and of varying the interval between the last feeding of this diet and slaughter were investigated. In general, uterine weight increased linearly with increased period of feeding and decreased linearly with delay in slaughtering. A feeding period of 4 days was satisfactory for comparisons based on uterine weight. If vaginal reactions were also to be utilized a feeding period of not less than 6 days was desirable.

In the second experiment the dose–response relationships of diethylstilboestrol, genistein and oestrogenic red clover were investigated. All materials were incorporated in the diet of mice, and the level of plant material was kept at 20 % of the mixed feed by adding inactive plant material where necessary. In general, either diethylstilboestrol or genistein could form a satisfactory standard for biological estimation of oestrogenic activity in red clover. Relative potencies based on uterine weight response and occurrence of vaginal opening and oestrus-type vaginal smears are given.

The biological estimation of oestrogenic activity in plant material involves a number of problems. Among the most important of these are: the choice of an appropriate response in a given test animal, a suitable duration of treatment and a satisfactory reference standard.

Biggers (1959), who has discussed the first of these problems, concluded that in mice vaginal cornification was more suitable than increase in uterine weight, because the former, unlike the latter, was a specific reaction to oestrogens. On the other hand, the uterine weight response has the advantage that it provides quantitative data. Flux, Munford & Barclay (1961) decided that, for initial screening purposes, uterine weight increase was more satisfactory because it was likely to show a response with low doses of oestrogens. Uterine weight increase has been commonly used to detect oestrogenic activity in plant materials (Cheng, Yoder, Story & Burroughs, 1954;

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Bickoff, Booth, Livingston, Hendrickson & Lyman, 1959; Kitts, Swierstra, Brink & Wood, 1959*a*, *b*).

When mice are used as test animals and the material to be tested is incorporated in the feed, the second problem amounts to determining satisfactory levels of incorporation of test material, duration of feeding the diet containing the test material and the best interval to allow between giving the last feed and slaughtering the animals. Kitts *et al.* (1959*a*), as a result of their studies, chose a 3-day feeding period with an 8-hour fast before slaughter with mice which received plant extract mixed with their normal diet. The level of incorporation could be varied quite widely. Bickoff *et al.* (1959), who found that dried ladino clover (*Trifolium repens*) could not be added to the meal mixture fed to their mice in quantities sufficient to give an adequate response in uterine weight, used plant extracts and allowed sufficient time (6–8 days) for the mice to consume an average of 10 g of food each. Uterine weights were lower if the test diet was withheld for 24 h before the mice were killed.

In studying the third problem Cheng *et al.* (1954) used four dose levels of diethylstilboestrol for comparative purposes while Kitts *et al.* (1959*a*) used a standard curve of uterine weights of mice treated with different levels of diethylstilboestrol for determining relative potencies of plant samples. A comparison of this curve with uterine weights resulting from use of dilutions of extracts from a red clover sample showed that the dose–response curve for the red clover sample fell reasonably well along the linear portion of the diethylstilboestrol dose–response curve. Bickoff *et al.* (1959) used a group of mice fed diethylstilboestrol at a single dose level in each of their experiments, and Pope, McNaught & Jones (1959) used two dose levels of oestradiol 17β for comparison with plant extracts, all material being injected subcutaneously.

In a previous study in this laboratory (Flux *et al.* 1961) a feeding period of 10 days was chosen arbitrarily and, although the food intakes of the mice were lowered when 20% freeze-dried plant material was incorporated in the meal mixture, the mice could gain weight slowly if they were not too small. They ate about 2·5 g each per day. In order to determine the best system for testing plant material for oestrogenic activity in our laboratory two experiments were conducted. In the first, ovariectomized mice received for varying periods a diet containing 20% freeze-dried red clover powder which was known to be oestrogenic, and were killed at varying times after the last feed was given. In the second, the dose–response relationships for diethylstilboestrol, genistein and red clover were investigated.

MATERIALS AND METHODS

Experiment 1

The mice were from the same colony (Flux, 1957), as those used previously for this type of work (Flux *et al.* 1961). Females with body weights in the range 8·5–13·9 g were ovariectomized at 21–23 days of age and fed a diet of one part dried buttermilk powder to two parts wholemeal flour for 2 days before starting the experiment. They were then fed the diet containing 20% freeze-dried red clover powder (mixed samples Aa 846 and rows 36 and 37, Table 2 in the paper by Flux *et al.* 1961) for the period

appropriate to their treatment, returned to the initial diet for a further 2, 3 or 4 days and then killed. Groups of control mice were treated similarly except that their experimental diet included 20% freeze-dried ladino clover (*T. repens*, sample C 1077, Table 1 in the paper by Flux *et al.* 1961) known to be non-oestrogenic. Five mice were used in each group, the experiment had a factorial design with an extra control group and mice were allotted to treatments at random. Details of the treatments are shown with the results in Table 1.

Table 1. *Effect of varying the feeding period of an oestrogenic diet and the time of slaughter on the uterine weight and occurrence of vaginal opening in immature ovariectomized mice* (*Expt.* 1)

Days on oestrogenic diet	Nil				2		
Days on control diet*	—	—	—	—	2	3	4
Day killed†	5	7	9	11	5	6	7
Mean uterine wt. (mg)	5·6	5·7	5·6	6·0	12·1	10·8	8·2
No. of mice showing vaginal opening	0	0	0	0	3	5	5
Days on oestrogenic diet	**4**			**6**			
Days on control diet*	2	3	4	2	3	4	
Day killed†	7	8	9	9	10	11	
Mean uterine wt. (mg)	19·7	14·2	10·0	20·7	16·8	17·1	
No. of mice showing vaginal opening	5	5	5	5	5	5	

* Days on control diet following oestrogenic diet and preceding slaughter.
† First day on treatment diet taken as day 1.
Each group contained five mice.

Experiment 2

The mice were from the same source, and they were ovariectomized at the same age and introduced to experimental diets in the same way as those in Expt. 1. Body weights ranged from 9·5 to 13·6 g. Ten mice, allotted at random, were used for each treatment group, the experimental feeding period for all the mice was 6 days and killing was on the 7th day.

The food mixtures were basically the same as those used in Expt. 1, the mice treated with genistein or diethylstilboestrol having the oestrogen added to the diet containing non-oestrogenic freeze-dried plant material. Thorough mixing was achieved by grinding the oestrogens with part of the dried buttermilk powder and mixing this carefully with the remainder of the food. Graded doses of red clover were obtained by using 20% freeze-dried red clover for the highest dose level and diluting this with non-oestrogenic plant material from the same lot as that used in the other diets. Four dose levels of red clover and five each of genistein and diethylstilboestrol were used, in each case lower doses were half the preceding dose. Details of treatments are shown with the results in Table 2.

In both experiments mice on the same diet were housed together and fed at the rate of 2·5 g per mouse per day. (Bickoff *et al.* 1959, found no reduction of variability of uterine weights through housing and feeding mice separately.) All food residues were collected, weighed each day and allowed for in determining food intakes. Mice

were checked once daily for the occurrence of vaginal opening and, in Expt. 2, for changes in the vaginal epithelium. Smears of vaginal contents were stained with methylene blue and classified according to the presence (non-oestrus, negative) or absence (oestrus, positive) of leucocytes (Biggers & Claringbold, 1954). At slaughter, uteri were excised, split lengthways, blotted and weighed immediately on a torsion balance.

Table 2. *Effect of various levels of diethylstilboestrol, genistein and red clover fed to mice on uterine weight and vaginal reactions (Expt. 2)*

Treatment code	Dose, μg,* planned (achieved)	Mean uterus wt., mg	Vaginal opening, no.	Positive† vaginal smears, no.
		Diethylstilboestrol		
A	0·009 (0·0086)	5·8	0	0
B	0·018 (0·0171)	6·4	0	0
C	0·036 (0·0347)	9·3	1	0
D	0·072 (0·0677)	11·2	1	0
E	0·144 (0·1377)	21·6	6	2
		Genistein		
F	1·5 (1·40)	6·6	2	0
G	3·0 (2·88)	7·8	7	1
H	6·0 (5·78)	11·6	9	7
I	12·0 (11·14)	22·8	10	9
J	24·0 (20·83)	34·3	10	10
		Red clover		
K	0·375 (0·340)	8·6	8	2
L	0·75 (0·70)	10·8	7	5
M	1·5 (1·37)	19·2	10	9
N	3·0 (2·77)	25·4	10	10

* Total dose for six day feeding period.

† The criterion of response was the disappearance of leucocytes from the smear (Biggers & Claringbold, 1954).

RESULTS

Experiment 1

As shown in Table 1, the mean uterine weights of the groups of control mice killed at different times during the experiment were very similar. The differences among them were not statistically significant, but the mean for all controls differed significantly from the mean for all treated mice ($P < 0{\cdot}001$). Among the treated mice, uterine weights increased with time on oestrogenic feed ($P < 0{\cdot}001$) and decreased with increasing time between last feed and slaughter ($P < 0{\cdot}001$). For each of these main effects there was a linear relationship between time and uterine weight ($P < 0{\cdot}001$); the non-linear component of variance was not significant. However, the interaction between these main effects probably represented real deviations ($P < 0{\cdot}1$) from these average linear relationships. Thus in Table 1, the mean uterine weight of the mice killed after 3 days on the control diet did not differ from that for mice killed after 4 days in the groups fed oestrogenic diet for 6 days. Also in the mice slaughtered after 2 days on the control diet the mean uterine weight of the groups fed on the oestrogenic diet for 6 days was not significantly different from that of the group fed this diet for 4 days.

Vaginal opening occurred in all mice fed oestrogenic clover with the exception of two mice in the group fed this diet for 2 days and killed after a further 2 days on control diet (Table 1). It was concluded that these mice had received sufficient oestrogen to cause vaginal opening, but were killed before the reaction occurred, since in all the other groups the mean time of vaginal opening was greater than 4 days. Because mice of this strain fed oestrogenic clover samples do not show oestrous changes of the vaginal epithelium before the day following vaginal opening it was decided to adopt a 6-day feeding period for the next experiment in which the incidence of positive vaginal smears was to be recorded.

Experiment 2

The details of treatments and results are shown in Table 2 and the dose–response lines for uterine weights are presented in Fig. 1. The daily food intakes of the mice were a little less than the 2·5 g which was necessary for them to get the full amount of oestrogen allotted to them. The differences arising from food refusals were about 7 % or less and did not increase with level of incorporation of oestrogen except with genistein where the percentage of food rejected for the highest dose level was 13·2. In plotting dose–response lines and calculating relative potencies the actual mean feed consumptions were used to calculate dose levels, all mice in each treatment group being regarded as having received the same dose.

The dose–response lines (Fig. 1) diverged at higher levels of the compounds tested and the slopes for all of them became steeper after doses corresponding to uterine weights of about 11 mg. However, the slopes of the lines were generally similar and it appeared worth while to find whether relative potencies could be calculated by bioassay methods (Finney, 1950). The results of the comparisons are shown in Table 3. It was necessary to transform uterine weight by taking logarithms to remove a relationship between subgroup means and variances existing in the original data. Doses of oestrogenic material were expressed in logarithms to make the response

curve to increasing doses more nearly linear and to make the data suitable for determining relative potencies by parallel-line bioassay methods. Where the variance in log uterine weight was significantly reduced by removing that part related (by regression) to differences in body weight, the calculations were based on the means and mean squares adjusted by covariance (Snedecor, 1956), except in the second assay in Table 2 where this adjustment introduced curvature of the dose-response due to a significant common quadratic component. The two quantal assays (the fourth and last in Table 3) covered only the dose ranges over which the number of positive reactions was changing.

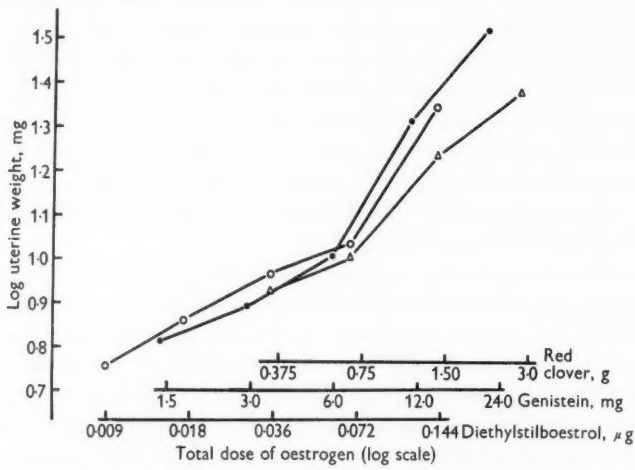

Fig. 1. The relationship between $\log_{10}$ uterine weight and doses of red clover, genistein and diethylstilboestrol. ○——○, Diethylstilboestrol; ●——●, genistein; △——△, red clover.

DISCUSSION

Experiment 1

The first experiment was preceded by an attempt, similar in object to Expt. 1, but differing from it in that each diet was fed in one or two cages only, mice on a particular combination of control and treatment diet being moved into and out of the appropriate cages as their treatment demanded. Judging from the low and variable food intakes and weight gains of the mice, these changes upset them. Because of this the experiment was repeated, as described in this paper, with the mice remaining in the same cages and the feed being changed as necessary. This time the food intakes and the growth of the mice were satisfactory with respect to levels and variability.

As far as increase in uterine weight was concerned the results showed that a 4-day feeding period was almost as good as a 6-day one, which is in accordance with the finding of Kitts *et al.* (1959*a*), who observed no increases in uterine weight in oestrogen-treated mice after the first 72 h. As in the present experiment Bickoff *et al.* (1959) found that uterine weights decreased with increased time after the last feed. The uteri of the mice fed oestrogenic clover for 6 days in the present experiment did not decrease in weight as much as the uteri of the mice fed for shorter periods.

Table 3. *Comparisons of oestrogenic activity of diethylstilboestrol, genistein and red clover (Expt. 2)*

Compounds compared	Response measured	Dose range	Relative potency	5 % fiducial limits	Lambda
Red clover and genistein	Uterus wt.	Clover KLMN Genistein FGHIJ	Assay not valid*	—	—
		Clover KLMN Genistein FGHI	6·42 mg genistein/g clover†	4·68–8·83 (73 %–183 %)	0·076
		Clover KLMN Genistein GHIJ	6·06 mg genistein/g clover‡	4·83–7·60 (80 %–125 %)	0·007
	Occurrence of positive vaginal smears	Clover KLMN Genistein GHI	7·33 mg genistein/g clover§	3·95–13·09 (54 %–179 %)	0·27
Red clover and diethylstilboestrol	Uterus wt.	Clover KLMN Dies. BCDE	6·72 μg dies./100 g clover‡	5·26–8·60 (78 %–128 %)	0·077
		Clover LM Dies. DE	8·36 μg dies./100 g clover‡‖	6·00–10·98 (72 %–131 %)	0·195
Genistein and diethylstilboestrol	Uterus wt.	Genistein FGHI Dies. BCDE	Assay not valid¶	—	—
		Genistein FGH Dies. BCD	$8{\cdot}93 \times 10^{-6}$ mg genistein/mg diethylstilboestrol‡	$(6{\cdot}58\text{–}12{\cdot}12) \times 10^{-6}$ (74 %–136 %)	0·130
		Genistein HI Dies. DE	$12{\cdot}06 \times 10^{-6}$ mg genistein/mg diethylstilboestrol‡‖	$(9{\cdot}43\text{–}15{\cdot}48) \times 10^{-6}$ (78 %–128 %)	0·166
	Occurrence of vaginal opening	Genistein FGHI Dies. CDE	$10{\cdot}3 \times 10^{-6}$ mg genistein/mg diethylstilboestrol§	$(2{\cdot}0\text{–}80{\cdot}0) \times 10^{-6}$	—

* Significant deviation from common linear regression.
† Covariance of uterus weight on body weight not used.
‡ Regression of uterus weight on body weight significant; adjusted means and mean squares used.
§ Doses with 0 or 100 % responses omitted.
‖ Doses D and E of diethylstilboestrol were those likely to be useful in later work with diethylstilboestrol as a standard.
¶ Significant common quadratic component.

A 4-day feeding period was satisfactory for comparisons based on uterine weight but it seemed best to feed oestrogenic clover for 6 days to allow more mice to undergo vaginal changes, in case this type of end point was useful, and to kill the mice 24 h after they were offered the last feed. Because some of the food given in the morning still remained uneaten in the early evening the time elapsing between the ingestion of the last of the clover and killing was probably only a few hours.

Experiment 2

In a recent paper not seen until the present work was completed, Bickoff and his colleagues (1960) showed that some alfalfa (*Medicago sativa*) meals used by them potentiated the activity of coumestrol while others acted as inhibitors. The authors are unaware of any evidence implicating other plant materials. In the present work genistein and diethylstilboestrol were added separately to diets containing freeze-dried non-oestrogenic ladino clover to provide the same proportion of plant material as in the red-clover-containing diets. The object was to equalize as far as possible the dilution effect of the plant material. Because no non-oestrogenic red clover was known it was impossible to use this species. The type of diet used would not be expected to equalize the effect of any oestrogen potentiators or inhibitors which might have been present. In determining levels of oestrogenic activity by biological means it would be difficult to distinguish between effects attributable to varying amounts of oestrogen and those due to potentiators or inhibitors, unless the latter caused dose–response curves to be very irregular when they were incorporated in diets containing graded doses of oestrogen of known activity. In the present work there was nothing in the appearance of the dose–response curves to suggest interference with the comparisons, but this evidence was far from conclusive.

The regressions fitted to all the data for uterine weight responses for genistein and clover deviated significantly from a common linear regression (Table 3). This could have been due to divergence or curvature, or to both types of deviation. When either of the extreme doses of genistein was omitted to give a (4 + 4) point comparison the deviations from common linear regression were not significant, this giving no indication that the relative activities differed significantly at different dose levels. The comparison based on occurrence of positive vaginal smears, using all dose levels which could contribute, again gave no indication that relative activity altered with dose level. The data in Table 2 show that doses of the two materials producing similar increases in uterine weight also produced similar numbers of open and cornified vaginas.

Pope, Elcoate, Simpson & Andrews (1953) found that the main oestrogenic isoflavone in their red clover was biochanin A (4′ methoxy genistein). Genistein is also present (Pope & Wright, 1954), and these two have similar levels of activity (Cheng *et al.* 1954). Provided that they also have a similar pattern of activity on different target organs, and no other oestrogenic compound contributed appreciably to the total observed activity, the result of the present experiment would not be surprising. However, Lyman, Bickoff, Booth & Livingston (1959) have detected a more active oestrogen, coumestrol, in red clover. The dose–response lines of coumestrol for different target organs do not appear to have been investigated. If it

occurred in the clovers used in the present experiment it must have been of minor importance or had a pattern of activity similar to that of genistein.

The comparison of genistein and red clover in Table 3, based on the two different end points, gave very similar estimates of relative activity of about 6·0 to 7·0 mg of genistein per gram of dry red clover. Because of loss of potency during storage of the red clover (Flux *et al.* 1961) this probably underestimated the activity of the dry matter of the fresh clover consumed by grazing animals. It is interesting to compare these estimates with those of about 4·0 to 8·0 mg of genistein per gram of dry leaf of subterranean clover made by Curnow (1954) and Curnow & Rossiter (1955). Firm conclusions about relative activity of red and subterranean clovers cannot be drawn, however, because of unknown losses with storage, unknown losses with extraction of subterranean clover (Curnow, 1954), the fact that the method used by Curnow & Rossiter (1955) was an approximate one and the possibility (Beck & Curnow, unpublished, cited by Curnow, 1954) that subterranean clover contained small quantities of an oestrogen more active than genistein.

Because of changes in the oestrogenic activity of clover samples with storage, a readily available standard of comparison such as diethylstilboestrol is likely to be useful. This substance has been used by Kitts *et al.* (1959*a*), who established a standard curve for mouse uterine weights resulting from the use of a range of doses. Such a method may be satisfactory if there are no changes in the oestrogenic sensitivity of mouse uteri with time. Changes in sensitivity to oestrogen have been observed, for example, with the mammary gland (Flux, 1957). The use of at least two doses of the standard in each experiment, as was done by Pope *et al.* (1959), is safer and leaves only the question of whether sensitivity to different types of oestrogen changed differentially with time.

In the present work a comparison was made between the uterine weight response curves for red clover and the four higher doses of diethylstilboestrol, the lowest dose being omitted to permit the comparison to be made on a (4+4) point basis. It was doubtful, in any case, whether this lowest dose was above the threshold for uterine weight increase. As shown in Table 3 there was no significant deviation from a common linear regression over this range, and the activity of the red clover in terms of diethylstilboestrol was estimated as equivalent to 6·7 μg per 100 g of dry red clover. Because it was likely that diethylstilboestrol doses 0·072 μg and 0·144 μg would be included as standards in later work, a comparison was made between the dose–response lines using the approximations achieved for those doses and the corresponding doses of red clover, 0·70 and 1·37 g. The relative activity was estimated as 8·4 μg of diethylstilboestrol per 100 g of dry red clover with 5 % fiducial limits 6·0–11·0 μg/100 g, which indicated that this type of standard would be useful for future work.

As shown in Table 2, the doses of diethylstilboestrol used, except the highest, had little effect on vaginal opening or cornification. The pattern of relative activity on different target organs was very different from that of red clover or genistein, doses causing considerable increases in uterine weight having relatively little effect on vaginal opening or cornification.

In terms of diethylstilboestrol equivalence the oestrogenic activity of the sample of red clover used in the present work (6·7 μg/100 g dry clover) was of the same order

as those of Kitts *et al.* (1959*b*), (up to 7·9 μg/100 g dry clover) who oven-dried their clover and extracted it with solvents. These workers adjusted mouse uterine weights for differences in body weight by taking them as a percentage of body weight, a method which, in addition to involving the assumption that a particular form of relationship between body and uterine weight exists, has the disadvantage of being prone to distort a dose–response curve when higher levels of oestrogen are given. This distortion arises because high levels of oestrogen can cause a reduction in food intake, resulting in lighter body weight, while still having large effects on uterine weight. In this situation the relationship between uterine weight and body weight would be quite different at different points on the same dose–response line and need not be the same at corresponding points of the dose–response lines for different oestrogens. Use of the analysis of covariance procedure to adjust uterus weight for variation in body weight has the advantage that no arbitrary assumption is made regarding the relationship between uterine weight and body weight.

The oestrogenic potency of genistein has been estimated by Carter, Smart & Matrone (1953) as $4{\cdot}44 \times 10^{-6}$ times that of diethylstilboestrol, while the approximate estimate of Cheng *et al.* (1954) was $1{\cdot}2 \times 10^{-5}$ times that of diethylstilboestrol. In the present study, over the greatest dose range for which such a comparison was valid, the estimate was $8{\cdot}93 \times 10^{-6}$, with 5 % fiducial limits $6{\cdot}58 \times 10^{-6}$ to $12{\cdot}13 \times 10^{-6}$, midway between the earlier estimates. The quantal comparison based on the occurrence of vaginal opening gave an estimate in good agreement with the above but, as would be expected from the data in Table 3, the reliability was very low.

The more important conclusions which can be drawn from this study appear to be that while red clover, genistein and diethylstilboestrol had dose–response curves for uterine weight which diverged a little and which were not entirely linear when both the response and dose were transformed by taking logarithms, either genistein or diethylstilboestrol might usefully be used as standards of comparison for red clover in different experiments. Genistein has the advantage of being similar to red clover oestrogens in relative activity on different target organs, but diethylstilboestrol is more readily available. Comparison of the dose responses of red clover and genistein gave no indication that red clover contained oestrogens with activities very different from those of genistein.

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Microscopic observations on the dispersibility of milk powder particles in some organic liquids

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Summary. By the application of different water-soluble organic compounds and some protein-denaturing reagents dissolved therein, several stages in the dispersion of milk powder particles can be distinguished. The particles first swell, and agglomeration or coalescence follows. However, no dispersion necessarily takes place at this stage, as indicated by the absence of casein micelles in the surrounding liquid. In the initial stage of self-dispersion, 'dispersion streams' emanating from the powder particles may be observed. In certain cases, particularly with spray dried skim-milk, thin membranes may be perceived around the swollen and dispersing powder particles. The dispersibility is dependent upon the fat content of the powder and the method of manufacture.

With propylene glycol and butylene glycol no dispersion of powder particles took place. However, the hydrogen-bond breaking reagents, urea, guanidine hydrochloride and sodium salicylate, as well as an anionic detergent, sodium dodecyl sulphate dissolved in propylene glycol, caused dispersion of powder particles, particularly in spray dried powders. The limited dispersibility of roller dried powders in water could be greatly enhanced by the same reagents.

With water-insoluble liquids enough moisture penetrated through the medium to cause the crystallization of lactose in powder particles.

For microscopic examination milk powders are usually mounted in a liquid medium in which they are more or less insoluble. However, they may also be investigated in air or in air saturated with water vapour (Mohr, 1960). In the latter case the vapour is quickly taken up by the powder particles, eventually causing their dispersion. The liquid mounting media may be water soluble (e.g. glycerol) or insoluble (e.g. paraffin oil). With either of these groups various reagents can be applied to the powder, provided they are soluble in the medium. For example, in differentiating between the fat and protein in powder particles the fluorescent stains phosphine and basic fuchsin are dissolved in glycerol, which acts simultaneously as a mounting medium (King, 1955).

The use of different mounting media, either alone or with various reagents dissolved therein, allows investigation of a range of properties of the milk powder particles. The present study deals with the effect of some water-soluble mounting media and some protein-denaturing reagents dissolved in them on the dispersibility of powder particles—a critical property in the reconstitution and recombination of

milk powders. The technique allows the dispersion to take place at a reduced, controlled speed so that intermediate stages in the process may be observed. Since the casein micelles set free from the powder particles during dispersion are readily perceivable by anoptral contrast microscopy, it provides a simple and convenient means of following the dispersion process. As might be expected, pronounced differences occur in the behaviour of different kinds of powders, depending upon the fat content, the manufacturing process and the storage conditions.

EXPERIMENTAL

The following kinds of milk powders were investigated: whole milk—spray and roller dried; skim-milk—ordinarily spray dried, low-heat spray dried, instantized (by the Blaw–Knox process), roller dried; buttermilk—spray and roller dried; ice cream mix—spray dried. These were commercial samples. As checked under the fluorescence microscope, spray dried whole milk exhibited a very fine dispersion of the fat, with no indication of any coalescence, whereas the roller dried product showed the usual picture—patches of coalesced fat on the periphery and within the powder particles. In skim-milk powders (spray and roller dried) almost no fat globules were perceivable. This was the case also with spray dried buttermilk. However, after reconstitution large numbers of minute fat globules became visible. In roller dried buttermilk quite large numbers of small fat globules were present, both on the periphery and within the powder particles. The following media, all very soluble in water, were used for mounting: ethylene glycol, propylene glycol (propane-1,2-diol), butylene glycol (butane-1,3-diol), glycerol, sorbitol (70% (w/v) aqueous solution) and saccharose (80% (w/v) aqueous solution). The following reagents were dissolved in propylene glycol: urea, guanidine hydrochloride, sodium salicylate and sodium dodecyl sulphate. Some observations also were made on the behaviour of milk powder in some water-insoluble mounting media, namely paraffin oil, castor oil and olive oil. A small quantity of powder was sprinkled on the surface of a drop of mounting medium on a slide, cautiously mixed, covered with a cover-glass and examined under the anoptral contrast microscope (King, 1959) at magnifications of $\times 450$ and $\times 1000$ (anoptral contrast objectives 20:1 and 45:1; pair of Kellner eyepieces, $\times 15$; binocular body, $\times 1{\cdot}5$.) Any stirring, rubbing or crushing of the powder particles was as far as possible avoided, so that the observations, which were made at room temperature, gave a picture of a more or less spontaneous dispersion (self-dispersion). With some of the mounting media the milk powder sprinkled on the surface spread out in a thin layer, which appreciably facilitated distributing the powder without stirring.

RESULTS

Effect of water-soluble mounting media on the dispersibility of milk powder particles

Varying forms and degrees of interaction between the powder particles and mounting medium could be observed. In some instances apparently no interaction took place, whereas with other mountants swelling occurred, accompanied by eventual agglomeration of powder particles. On swelling, the casein micelles and fat globules in the powder particles became more distinctly visible, and in spray dried skim-milk, particularly in the low-heat product, a thin membrane appeared around

the powder particles. Such a membrane sometimes persisted until the swollen particles were almost entirely disengaged. On dispersing the milk powder in water the membranes appeared as 'bags'. The agglomerated powder particles may retain their outlines, or they may coalesce to a uniform mass. However, swelling and agglomeration are not necessarily accompanied by the dispersion of the powder particles, and under the microscope the mounting medium between powder particles then remains empty.

Table 1. *Dispersion of milk powder particles in different mounting media*

Milk powder	Ethylene glycol	Propylene glycol	Propylene glycol + urea (4 M)	Propylene glycol + guanidine HCl (4 M)	Propylene glycol + Na salicylate (2 M)
Whole milk, spray	−	−	+++	++++	++++
Whole milk, roller	−	−	+	+	+
Skim-milk, spray, ordinary	+++	−	+++	++++	++++
Skim-milk, spray, low-heat	+++	−	+++	++++	++++
Skim-milk, instant	+++	−	+++	++++	++++
Skim-milk, roller	−	−	+	++	++
Buttermilk, spray	+++	−	+++	+++ (1 M)	+++ (0·5 M)
Buttermilk, roller	++	−	++	+	−
Ice cream mix, spray	++	−	++	++	++

Milk powder	Propylene glycol + Na dodecyl sulphate (0·34 M)	Butylene glycol	Glycerol	Sorbitol (70 % (w/v))	Saccharose (80 % (w/v))
Whole milk, spray	+	−	−	−	−
Whole milk, roller	+	−	−	−	−
Skim-milk, spray, ordinary	++	−	++	++	+++
Skim-milk, spray, low-heat	+++	−	++	++	+++
Skim-milk, instant	+++	−	+	+	++
Skim-milk, roller	−	−	+	−	−
Buttermilk, spray	++	−	−	++	++
Buttermilk, roller	+	−	−	+	+
Ice cream mix, spray	+++	−	−	−	+

−, No dispersion (dispersion medium between powder particles empty); +, slight dispersion (some few casein micelles and fat globules in dispersion medium); ++, fairly strong dispersion (quite a number of casein micelles and fat globules in dispersion medium; quite a number of powder particles still left); +++, strong dispersion (dispersion medium full of casein micelles and containing many fat globules; some few powder particles still left); ++++, almost complete dispersion (dispersion medium densely crowded with casein micelles and containing many fat globules; almost no powder particles left).

During the dispersion proper of powder particles the mounting medium becomes more and more densely crowded with the casein micelles and fat globules, and obviously increases considerably in viscosity since no Brownian movement of the casein micelles is observable. The dispersion starts almost immediately with the making of the specimen, setting in on the periphery of the powder particles. At the beginning the liberated casein micelles form 'dispersion streams' in the mounting medium. However, after some time these streams dissipate into a uniform mass of crowded casein micelles.

The extent to which the different milk powders are dispersible in the water-soluble mounting media investigated is given in Table 1. Ethylene glycol, the first member of the dihydric alcohols, did not disperse either whole milk powders or roller dried skim-milk powder, whereas the next two members, propylene glycol and butylene glycol did not disperse any of the powders. Glycerol, the first member of the trihydric alcohols, showed less dispersing ability than ethylene glycol. The hexahydric alcohol sorbitol and the disaccharide saccharose were slightly more active than glycerol.

It is of interest that appreciable dispersing ability could be conferred on propylene glycol by dissolving in it some protein-denaturing substances. The substances used, urea, guanidine hydrochloride, sodium salicylate and sodium dodecyl sulphate, were quite soluble in propylene glycol (up to 2–4 M), but only slightly soluble in butylene glycol. The highest dispersing ability was displayed towards the spray dried powders. The concentrations involved were 4 M for urea and guanidine hydrochloride, 2 M for sodium salicylate and 0·34 M for sodium dodecyl sulphate. However, the dispersing ability already became apparent at lower concentrations (1–2 M). Among the hydrogen-bond breaking reagents, guanidine hydrochloride was more effective than urea and less effective than sodium salicylate. The same order of effectiveness was shown by these reagents on interacting with casein in skim-milk and in lactic and rennet clots (King, 1960). On dispersion of spray dried whole milk, buttermilk and ice cream mix no coalescence of fat globules took place.

The photomicrographs Pl. 1, *a–d*, Pl. 2, *a–d*, Pl. 3, *a–c*, and Pl. 4, *a*, *b*, depict the interaction phenomena between the powder particles and mounting medium (swelling, agglomeration, coalescence, dispersion proper, 'dispersion streams') for spray dried whole milk, skim-milk (low-heat) and buttermilk, as influenced by different mounting media and some reagents dissolved in propylene glycol.

The dispersibility of milk powder in saccharose solution is of importance in the manufacture of sweetened condensed milk by the recombination of skim-milk powder, sugar and butterfat. Pont (1960) found that spray dried skim-milk mixed readily with strong sugar solutions (44 lb sugar in 25 lb water), but gave a product of mealy texture, which after reconstitution with water and centrifuging gave a heavy sediment of undissolved powder particles. Reducing the sugar content to 11 lb per 25 lb of water gave an adequate solution of milk powder (the rest of the sugar being added afterwards). In the present investigation spray dried whole milk was almost non-dispersible in 80 % (w/v) sucrose solution. In 40 % (w/v) solution the dispersion was fairly strong, but even in 16 % (w/v) solution there was quite a number of undispersed particles, which, however, were greatly swollen. The dispersion took place much more easily with spray dried skim-milk, which in 80 % (w/v) sucrose solution exhibited strong dispersion, while in 40 % (w/v) solution the dispersion was almost complete.

Effect of reagents on the dispersibility of roller dried powders in water

In the present observations, roller dried powders mounted in distilled water exhibited a rather moderate dispersibility, whereas spray dried powders, particularly skim-milk powder, were much more dispersible, which is in agreement with the generally recognized fact that on reconstitution the former are less soluble than the

latter. However, when roller dried powders were mounted in aqueous solutions of protein-denaturing reagents, quick and abundant swelling and dispersion of powder particles took place. Table 2 shows the concentrations needed to bring about this effect. Here again the reagents arranged themselves in dispersing ability in the same order as above.

The calcium-binding substances, disodium ethylenediaminetetra-acetate in 0·25 M solution and sodium citrate in M solution, did not exert a dispersing action.

Table 2. *Concentrations of protein-denaturing reagents needed to bring about a quick and copious swelling and dispersion of the roller dried milk powders in water*

Reagent	Whole milk powder	Skim-milk powder	Buttermilk powder
Urea	6 M	4 M	3 M
Guanidine hydrochloride	4 M	2 M	2 M
Sodium salicylate	2 M	1 M	—
Sodium dodecyl sulphate	0·1 M	0·08 M	0·08 M

Penetration of moisture through water-insoluble mounting media

The specimens of milk powders mounted in water-insoluble media (e.g. paraffin oil), after standing for some days at room temperature and humidity, show a narrow crystalline zone on their periphery. The formation of this zone is apparently due to slow penetration of moisture through the medium to the powder particles, where it causes the crystallization of lactose. This phenomenon can be enhanced appreciably by keeping the specimen in an atmosphere saturated with water vapour.

The specimens were made in conventional haemocytometers 100 μ thick and kept in a desiccator above a layer of water (at room temperature). Table 3 gives some data on the extent of water penetration with different oils, while Pl. 4, *c*, represents a photomicrograph of the lactose crystallization zone.

Table 3. *Width of the crystallization zone of lactose in milk powder mounted in different oils and kept in moisture-saturated atmosphere*

	Spray dried whole milk		Spray dried buttermilk
Mounting medium	4 h	22 h	24 h
Paraffin oil	70μ	250μ	200μ
Castor oil	200μ	1000μ	700μ
Olive oil	—	—	850μ

The higher penetrability of moisture in castor oil and olive oil, as compared with paraffin oil, is apparently due to a more hydrophilic character of the former. With water-soluble butylene glycol as mounting medium, lactose was crystallized in the whole counting chamber—width 6300 μ—in 4 h. With the water-insoluble mounting media the lactose crystals generally occurred within the powder particles, whereas with butylene glycol they appeared mostly outside the powder particles. Seemingly the amount of moisture penetrated into butylene glycol was large enough to dissolve the lactose from the powder particles and enable it to migrate into the mounting medium, where it crystallized.

DISCUSSION

During the drying of milk, the casein micelles are densely crowded in a medium consisting of highly concentrated lactose in a glassy state and containing the concentrated milk salts and the fat globules. Undoubtedly the casein micelles come into contact with each other, the bonding strength being dependent upon the manufacturing technique. It is obvious that during roller drying with its higher temperatures of longer duration, as compared with spray drying, a stronger interaction between the micelles may be expected. Unfavourable storage conditions may further enhance the sticking together of micelles.

The reconstitution process, on the other hand, aims to separate the casein micelles, re-establishing a dispersion state as similar as possible to that in the original milk. The dispersion of a powder particle comprises the dissolution of lactose glass and dispersion proper of the casein micelles (and fat globules) by the dispersing medium. The prerequisite, of course, is a ready access of the dispersing medium to the powder particles, which in turn involves an adequate wettability and penetrability of powder particles. As suggested by the present study, it is the hydrogen bond which is mainly involved in the bonding of casein micelles. However, the hydrophobic bonds may also play a role, as indicated by the dispersing action of sodium dodecyl sulphate. According to Kauzmann (1959) urea and guanidine hydrochloride are also able to weaken hydrophobic bonds as well as hydrogen bonds. The bonding strength is less in spray dried powders, where the hydrogen-bond breaking substances were already active in non-aqueous solution. It is much more pronounced in roller dried powders, where these reagents displayed activity first in aqueous solution (cumulative action of water and reagent). The dispersing ability of reagents appeared in the same order, as previously observed in their interaction with casein in skim-milk and in lactic and rennet clots (King, 1960). However, there was no response in roller dried powders towards the calcium-sequestering reagents.

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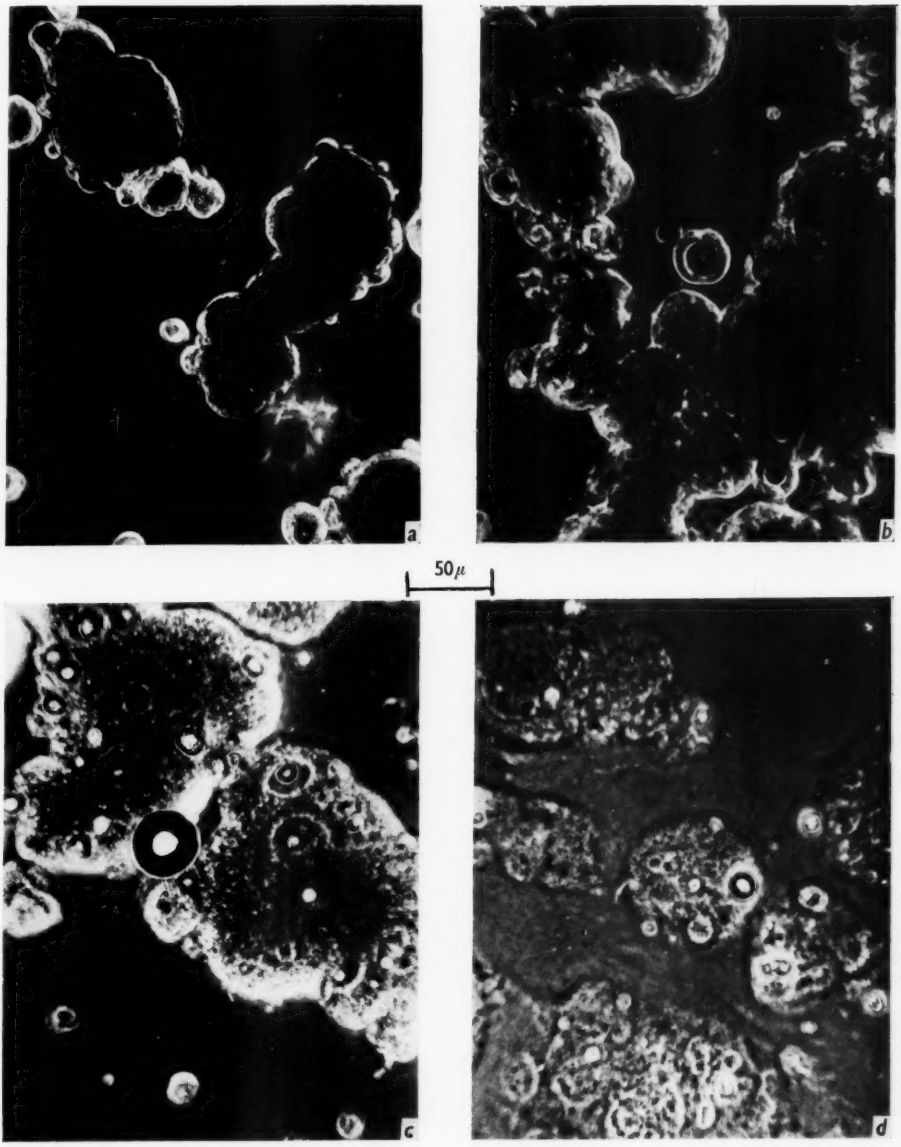

N. KING AND P. D. SHIMMIN

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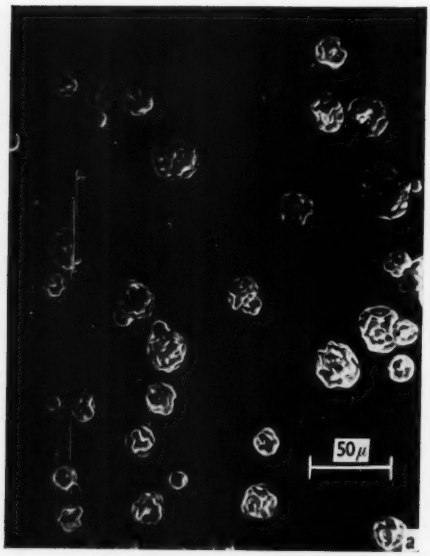
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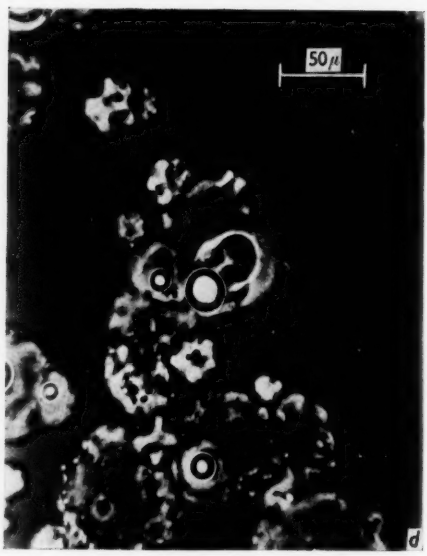
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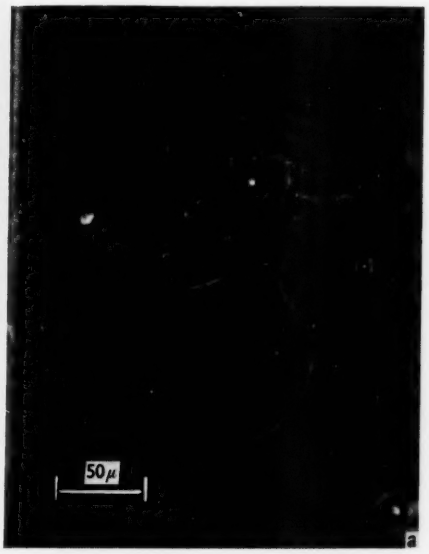
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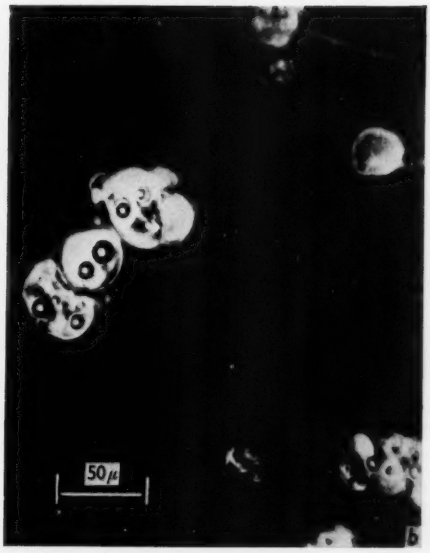
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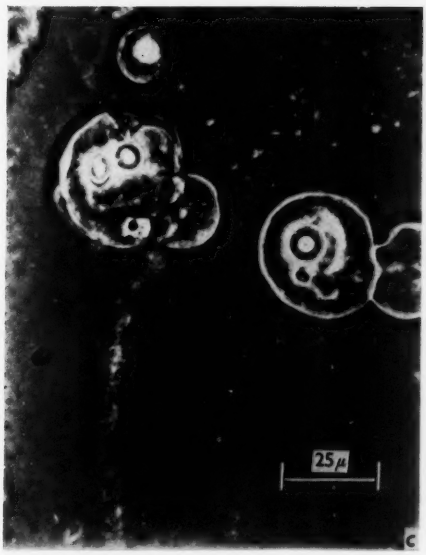
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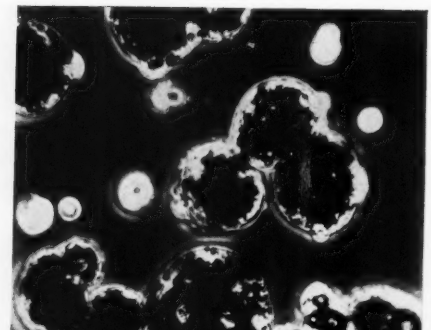

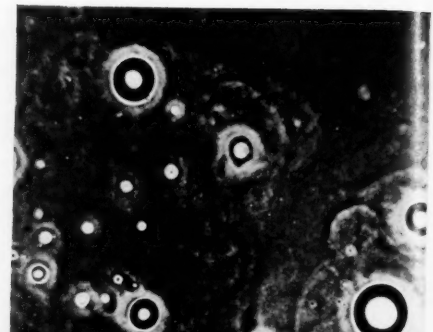

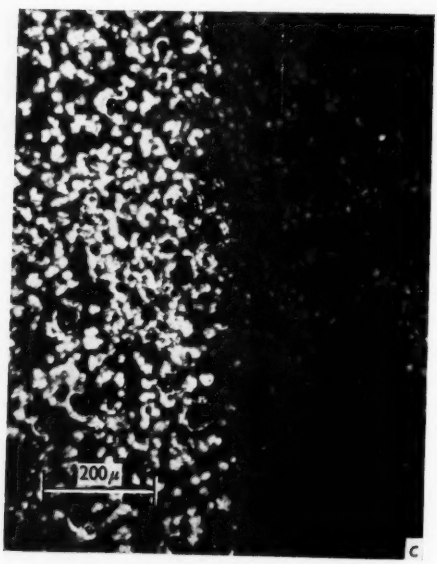

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c. Lov

a. Spr
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light.

EXPLANATION OF PLATES

Plate 1

a. Spray dried whole milk in butylene glycol. Slight swelling; no dispersion.

b. Spray dried whole milk in propylene glycol. Slight swelling; partial agglomeration; no dispersion.

c. Spray dried whole milk in 4 M-solution of urea in propylene glycol. Strong swelling; fairly strong dispersion.

d. Spray dried whole milk in 4 M-solution of guanidine hydrochloride in propylene glycol. Strong dispersion.

Plate 2

a. Low-heat spray dried skim-milk in butylene glycol. No swelling; no dispersion.

b. Low-heat spray dried skim-milk in propylene glycol. Slight swelling and agglomeration; no dispersion.

c. Low-heat spray dried skim-milk in propylene glycol (at higher magnification). Slight swelling; no dispersion; membrane around the powder particle.

d. Low-heat spray dried skim-milk in 2 M-solution of urea in propylene glycol. Swelling; coalescence, fairly strong dispersion.

Plate 3

a. Low-heat spray dried skim-milk in 4 M-solution of urea in propylene glycol. Almost complete dispersion; membranes.

b. Low-heat spray dried skim-milk in glycerol. 'Dispersion streams.'

c. Low-heat spray dried skim-milk in glycerol (at higher magnification). 'Dispersion streams.'

Plate 4

a. Spray dried buttermilk in butylene glycol. No dispersion.

b. Spray dried buttermilk in 4 M-solution of urea in propylene glycol. Almost complete dispersion.

c. Low-heat spray dried skim-milk in castor oil after 16 h standing in humid atmosphere. Polarized light. Powder particles with crystallized lactose bright.

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Gas packing milk powder with a mixture of nitrogen and hydrogen in the presence of palladium catalyst

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(*Received* 24 *May* 1961)

Summary. Whole and skimmed milk spray dried powders have been gas packed by two methods, and the chemical and physical changes taking place during storage at 17 and 37 °C for two years have been measured. The conventional method of double gas packing with nitrogen, with an interval of several days for desorption, was used as a control for a new method employing a single gas packing with a mixture of nitrogen (90 %) and hydrogen (10 %) plus the inclusion within the can of a solid catalyst pellet containing palladium.

The new system was more effective in removing oxygen and in maintaining an almost oxygen free atmosphere within the cans. Used on a commercial scale it would have several important advantages over the conventional method, and both whole and skimmed milk powders packed in this way would probably be usable after storage for 10 years at normal temperatures.

Because of the low general level of oxygen in all the cans packed by both methods and because of the low moisture content there was little difference in the keeping quality of the powders. What little there was, however, was in favour of the new method.

The most effective method of extending the storage life of milk powder is to pack it in an airtight container from which the air has been removed and replaced by a chemically inert gas such as nitrogen, the process commonly known as gas packing. This is particularly true for whole milk powder where the storage life is governed by the rate of oxidation of the unsaturated fats and the consequent development of objectionable flavours (Lea, Moran & Smith, 1943). The advantage of gas packing skimmed milk powder of normal moisture content (about 3 %) is much less but it has been found to be worth while in preventing the development of stale flavours, especially where the storage period might be prolonged or the storage temperature high (Henry, Kon, Lea & White, 1948).

The technique of gas packing is simple and, so long as the containers are airtight, the oxygen content can easily be reduced from the 21 % present in air to 1 % or less

immediately after the operation is finished. With spray dried powder, however, this diminution is limited mainly to the atmosphere surrounding the hollow powder particles and as much as 28 days may need to elapse before equilibrium is established between the gases within and around the particles, although most of the change will usually be complete after 7–10 days. During this desorption period the oxygen content within the can may rise to as much as 5 %, considerably above the level of 1–1·5 % required to ensure optimal keeping quality (Lea *et al.* 1943). When oxygen levels of the order of 1 % are needed there is no alternative in the usual gas packing technique but to store the powder after the initial gas packing long enough for desorption to take place and then to gas pack the cans a second time. This is costly and time consuming, requires considerable storage space, increases the handling of the cans and is unsuitable for normal factory routine. For these reasons a process is required which will remove the desorbed oxygen as it is liberated and, if possible, allow more latitude in the initial gas packing.

The publication by King (1955) of a method of removing small amounts of oxygen by combination with hydrogen at normal temperatures in the presence of a palladium catalyst, suggested a simple alternative to the conventional method of gas packing described above, and one which might permit successful storage of milk powder for considerable periods. A comparison of the two methods has therefore been made, using commercial, freshly manufactured spray dried whole and skimmed milk powders stored at 17 and 37 °C for a little more than 2 years.

EXPERIMENTAL

The milk, either whole or skimmed, was heated at 195 °F for 15 sec and concentrated to 42 % total solids before being spray dried in a centrifugal disk type machine. The whole milk powder contained 28·2 % fat and 2·2 % moisture, the skimmed milk powder 0·2 % fat and 2·4 % moisture. Both powders were packed 2–3 weeks after manufacture in tinplate cans to within about 0·5 cm of the top, giving a weight of 127 g of powder in a volume of 225 ml. In addition, a few larger cans of skimmed milk powder were packed to test the effectiveness of the nitrogen-hydrogen-palladium system, and these contained 3·4 kg in a volume of 4·22 l.

Gas packing was done in a factory-size cabinet, the cans gas packed by the conventional method receiving three successive cycles of evacuation and nitrogen filling before the brogue holes were soldered over. These cans were allowed to stand for 8 days for oxygen desorption from the powder to take place and then they were gas packed a second time with oxygen free nitrogen containing less than 0·01% oxygen.

The palladium catalyst was incorporated in a small cylindrical pellet of inert carrier (approximately 3 × 3 mm in size of the type used industrially for gas purification) loosely wrapped in filter paper and attached to the underside of the lid before closing the can. The large cans containing 3·4 kg of skimmed milk powder received two palladium pellets, one at each end. To enable both nitrogen and hydrogen to be used in the same gas packing operation the cabinet was modified by the addition of a small pressure cylinder, to contain the desired amount of hydrogen gas, connected to the cabinet and the gas lines by a three-way tap. These cans therefore received only a

single gas packing which resulted initially in an atmosphere within of 10 % hydrogen and 90 % nitrogen.

Cans gas packed by both systems were examined, as described below, prior to storing half of each set at 17 and 37 °C; cans were subsequently withdrawn for examination at intervals of 2 months.

Examination of stored powders

The percentage of oxygen and carbon dioxide in the can was determined on a sample abstracted with suitable equipment through a puncture hole before the can was opened. After opening the can, the powder was well mixed, placed in airtight bottles and analysed at once for moisture content (British Standards Institution, 1951), peroxide value (Loftus Hills & Thiel, 1946) and solubility in water (Howat, Smith, Wright & Waite, 1939). The taste of the reconstituted powder (at 12·5 % total solids for whole milk powders and 9 % for skimmed milk powders) was judged independently by a panel of five people, using fresh milk, either whole or skimmed, for comparison. A simple descriptive grading scale of 0 to 5 was used for scoring and good agreement was usually obtained between tasters. The arithmetic mean of the five scores was recorded at each examination.

RESULTS

Gas analysis

The cans were gas packed in Aberdeen and sent to Ayr for storage and analysis, which introduced a period of 12 days between final gas packing and the first gas analysis. The percentage of oxygen or carbon dioxide in the can was converted to mg/100 g of powder at 17 °C using a factor for the density of whole milk powder of 1·33 and of skimmed milk powder of 1·46 (Lea & Gane, 1946).

(*a*) *Whole milk powder*

The cans which were gas packed in the conventional manner with nitrogen contained initially 0·8 % of oxygen in the headspace gas (equivalent to 1·084 mg of oxygen/100 g of powder) whilst those gas packed by the new method with the mixture of nitrogen and hydrogen contained only 0·1 % of oxygen (0·135 mg/100 g of powder). There was thus a distinct advantage in favour of the new method before the storage period commenced. Neither set of cans contained any measurable amount of carbon dioxide at this time.

The absorption of oxygen and the generation of carbon dioxide during storage for 112 weeks at the two temperatures are shown in Fig. 1, where it can be seen that changes in gas composition began only after storage for 24 weeks. The absorption of oxygen was more rapid in the conventionally gas packed cans, where a greater amount was available, and at 37 °C approximately 80 % of that present at the beginning of the storage period was absorbed during the next 8 weeks, although a further 80 weeks was required to remove the remainder. At 17 °C only half the available oxygen in the conventionally gas packed cans had been absorbed after 112 weeks, there being little change after about the fiftieth week. In the cans gas packed with the mixture of nitrogen and hydrogen, storage temperature had little apparent effect on oxygen

absorption, although the difficulty of accurately measuring very low oxygen contents in the gases within closed cans is shown by some irregularity of the analytical values at both temperatures. All the small amount of available oxygen had been removed in these cans towards the end of the storage period.

The evolution of carbon dioxide from all the powders was clearly governed more by the storage temperature at the low oxygen concentrations prevailing than by the method of gas packing. This is to be expected since the Strecker degradation of the sugar–protein compound formed during the production of milk powder, to carbon dioxide and aldehyde, the major source of the liberated carbon dioxide, is sensitive to temperature (Hodge, 1953).

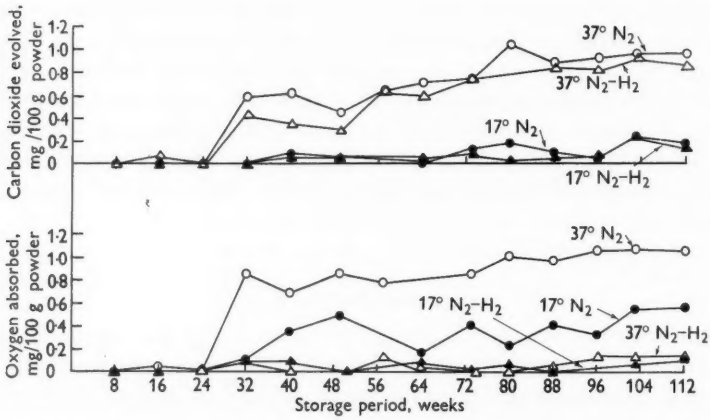

Fig. 1. Changes in oxygen and carbon dioxide contents in gas packed cans of whole milk powder. —○——○—, Nitrogen at 37 °C; —●——●—, nitrogen at 17 °C; —△——△—, nitrogen–hydrogen at 37 °C; —▲——▲—, nitrogen–hydrogen at 17 °C.

(*b*) *Skimmed milk powder*

Prior to storage, the cans gas packed in the conventional manner with nitrogen contained 0·6 % oxygen (equivalent to 0·8 mg oxygen/100 g powder), but during the next 8 weeks there was an increase to 1·35 % (1·86 mg/100 g powder). The latter value was substantiated in the three subsequent analyses and appeared to be valid. It must be concluded that insufficient time was allowed for complete oxygen desorption of this powder between the first and second gas packings, although in preliminary trials with a previous batch of powder from the same plant 8 days had proved sufficient. It is, however, known (Lea *et al.* 1943) that there can be considerable variation in the rates of desorption of different batches of powder from the same plant and this appears to have happened. For the purpose of calculating oxygen absorption during storage the higher value has been used. The oxygen content of the cans gas packed with the mixture of nitrogen and hydrogen was much lower, 0·13 % (0·19 mg/100 g powder) and similar to that of the cans of whole milk powder treated in the same way. For long-term storage the advantage was thus again strongly in favour of the cans containing the palladium catalyst.

Fig. 2. shows the changes in gas composition within the cans of skimmed milk

powder during storage. The chief difference between these results and those in Fig. 1 is the absence of any induction period in oxygen absorption by the skimmed powder at 37 °C in the conventionally gas packed cans. The rate of absorption, however, in the virtual absence of fat, was lower than in the whole milk powder, only 58 % of the available oxygen having been absorbed after 32 weeks and 86 % after 112 weeks. The cans of this set (nitrogen pack) stored at 17 °C absorbed only some 20 % of the available oxygen by the end of the storage period, whereas in the same time the corresponding whole milk powders had absorbed 52 % (Fig. 1). Skimmed powder stored in an atmosphere of nitrogen and hydrogen absorbed about half of the little oxygen available, irrespective of the temperature of storage.

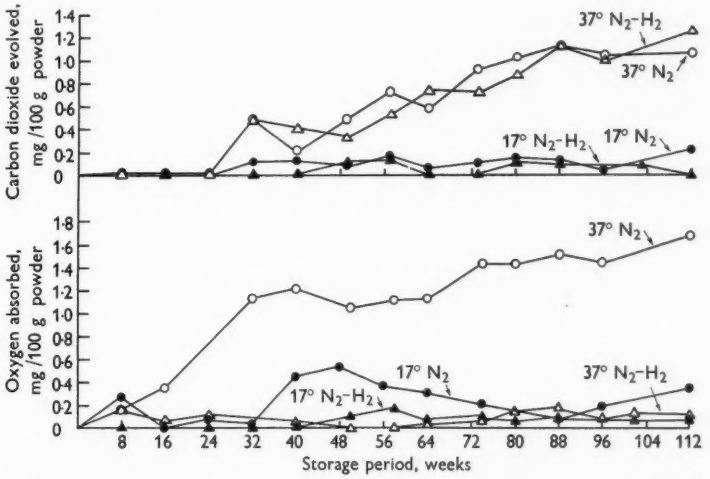

Fig. 2. Changes in oxygen and carbon dioxide contents in gas packed cans of skimmed milk powder. —○——○—, Nitrogen at 37 °C; —●——●—, nitrogen at 17 °C; —△——△—, nitrogen–hydrogen at 37 °C; —▲——▲—, nitrogen–hydrogen at 17 °C.

The amount of carbon dioxide evolved by the skimmed milk powders stored at 37 °C was again very similar for both types of gas packing and little different from that evolved by the whole milk powders. At 17 °C very little carbon dioxide was evolved by any of the powders.

Peroxide values in the whole milk powder during storage

There was no development of peroxide in the powder gas packed by either method during storage, either at 17 or 37 °C. From an initial value for both powders of 1 m-equiv. of oxygen/kg fat, there was a slow progressive fall to zero after 112 weeks.

Taste of the reconstituted powders

The development of the tallowy flavours associated with increasing oxidation of the fat was, as would be expected at such low levels of available oxygen, slow and small. Similarly, since the improvement obtained in the keeping quality of skimmed milk powder by gas packing is, at any time, not very great (Henry *et al.* 1948), it was

not surprising that there was no difference in taste between the powders gas packed by the two methods. Only in the powders stored at 37 °C was there a very slight advantage in favour of the new method of gas packing, and all powders were palatable after storage for 112 weeks. There was no colour development in any of the powders.

Mean flavour scores at intervals during the storage period are given in Table 1 (whole milk powders) and Table 2 (skimmed milk powders).

Table 1. *Taste scores for whole milk gas packed powders at* 17 *and* 37 *°C*

Score 0 = no off-flavour; 1 = tallowy flavour just distinguishable; 2 = slight tallowy flavour, powder usable; 3 = definite tallowy flavour, powder unusable.

Type of gas packing	Storage temperature, °C	Taste score after storage for 0–16 weeks	17–40 weeks	41–60 weeks	61–80 weeks	81–112 weeks
Nitrogen	17	0·4	0·6	0·9	1·1	0·7
Nitrogen–hydrogen	17	0·4	0·6	0·7	0·7	0·6
Nitrogen	37	0·5	1·0	1·1	1·4	1·2
Nitrogen–hydrogen	37	0·5	0·7	0·8	1·1	1·0

Table 2. *Taste scores for skimmed milk gas packed powders at* 17 *and* 37 *°C*

Score 0 = no off-flavour; 1 = cardboard flavour just distinguishable; 2 = slight cardboard flavour, powder usable; 3 = definite cardboard flavour, powder unusable.

Type of gas packing	Storage temperature, °C	Taste score after storage for 0–16 weeks	17–40 weeks	41–60 weeks	61–80 weeks	81–112 weeks
Nitrogen	17	0·5	0·8	0·9	0·8	0·9
Nitrogen–hydrogen	17	0·4	0·6	0·4	0·5	1·1
Nitrogen	37	0·6	1·0	0·9	1·3	1·9
Nitrogen–hydrogen	37	0·5	1·1	0·7	1·1	1·6

Solubility of the powders in water

The solubility indices of the powders at the start of the experiment were: whole milk, 100 at both 20 and 50 °C; skimmed milk, 99 at 20 °C and 100 at 50 °C. These values remained unchanged throughout the storage period at both 17 and 37 °C.

Skimmed milk powder stored in larger quantity

Cans containing 3·4 kg of powder, gas packed with the mixture of nitrogen and hydrogen in the presence of two palladium pellets and stored at 37 °C were examined initially and at 6-monthly intervals for 2 years. The cans were virtually oxygen free throughout the whole period, the analyses varying between 0·03 % (0·02 mg of oxygen/100 g of powder) and zero. There was a steady generation of carbon dioxide during the storage period, amounting in all to 0·94 mg/100 g of powder after 112 weeks, a value not unlike that (1·2 mg/100 g) found in the smaller cans. There was only very slight deterioration of the powder in taste and none in solubility.

DISCUSSION

The results obtained in this experiment show clearly that gas packing milk powder with a mixture of nitrogen and hydrogen in the presence of a solid catalyst to promote the combination of hydrogen and oxygen was more effective in removing the residual and desorbed oxygen than double gas packing with pure nitrogen alone. It is true that when the conventional method, with double gas packing, is efficiently applied the absolute difference in the amounts of oxygen available to the powder will be small and likely to be of importance in keeping quality only after a storage period of several years. But the advantage of the new method lies much more in the simplicity of its application and in the elimination of the waiting period for oxygen desorption. On a factory scale, the necessity to provide storage space for periods which, as seen from the present results, may need to be greater than 1 week, the increased handling of the cans, the extra gassing and the general additional possibility of error, has always been an irksome and costly concomitant of double gas packing. Although in the present experiment the gas packing cabinet was structurally modified to accommodate the hydrogen supply, this would be unnecessary in routine work where a commercial mixture of nitrogen and hydrogen could be used in existing cabinets. It would appear from our results that the new method would be equally effective if used in the normal range of can sizes used in the milk powder industry.

There is no danger that the water formed by the combination of hydrogen and oxygen would endanger the keeping quality of the powder. If it is assumed that desorption would, in the absence of hydrogen and palladium, raise the oxygen content within the can to 10%, an unlikely high value, the increase in moisture content caused by the reduction of this oxygen to water would be slightly less than 0·02%, i.e. within the accuracy of the moisture determination.

The advantage of gas packing in prolonging the life of milk powder, particularly of whole milk powder, is well known and as the present results are very similar to those previously obtained (Lea *et al.* 1943; Henry *et al.* 1948) they require little discussion. If a temperature coefficient for the development of tallowiness is taken as 2 for each 10 deg C rise in storage temperature (Lea *et al.* 1943), the results from the powders stored at 37 °C indicate that a useful storage life of at least 9–10 years at a temperature of 17 °C should be possible for both whole and skimmed milk powder. At higher storage temperatures it is likely that the limiting factor in keeping quality would be the gradual accumulation of carbonyl-containing bodies (Bassette & Keeney, 1960) resulting from the breakdown of the lactose–protein compound formed in the milk during the preheating and evaporating stages of powder manufacture. The steady accumulation of carbon dioxide at 37 °C in cans which were initially almost free from oxygen is evidence of this type of deterioration.

The authors wish to thank Mr E. J. Rolfe for packing the powders and for collaboration in preliminary trials, and Miss M. H. King for her assistance with the analytical work.

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The occurrence of pyridoxamine phosphate in milk

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Summary. The presence of pyridoxamine phosphate in extracts of freeze-dried raw and evaporated milks has been demonstrated by separation and identification of the vitamin B_6 active compounds by chromatography and electrophoresis on paper. Its presence in the milk extracts is the cause of the higher values for vitamin B_6 activity measured with *Streptococcus faecalis* as compared with those obtained with *Str. faecium* or *Saccharomyces carlsbergensis*. This is because *Str. faecalis* can utilize the phosphate for growth as readily as free pyridoxamine, whereas *Str. faecium* and *S. carlsbergensis* cannot. The mild acid treatment used for extracting the vitamin B_6 active compounds from the milk samples for microbiological assay was found to be insufficient to hydrolyse the pyridoxamine phosphate. Further treatment of the acid extracts with intestinal phosphatase released the pyridoxamine from its phosphate and increased the vitamin B_6 activity measured with *S. carlsbergensis* and *Str. faecium* so that the total vitamin B_6 activities of the freeze-dried raw and evaporated milks measured microbiologically, were then in agreement with the values found in previous tests with chicks and rats.

Pyridoxamine phosphate could only be detected in small amounts in a sample of fresh milk. The possibility that more of it was formed during the processing and storage of the freeze-dried samples is discussed.

In the course of an investigation on the effect of heat treatment on the vitamin B_6 content of milk, it was consistently observed that more vitamin B_6 activity was measured with *Streptococcus faecalis* R, than with *Str. faecium* Ø51 as the test organism (Gregory, 1959). Since pyridoxal and pyridoxamine have about equal activities and pyridoxine is inactive for both organisms in growth tests, it seemed that the stimulatory factor for *Str. faecalis* was something other than one of the vitamin B_6 analogues. The presence of vitamin B_6 phosphates in the extracts was not suspected since, in preliminary tests with fresh milk and a sample of National Dried Milk, prolonging the acid extraction treatment and using a higher temperature (in order to hydrolyse the phosphates) did not increase the vitamin B_6 activity measured with *S. carlsbergensis*. Therefore it was assumed that milk contained mainly free pyridoxal and pyridoxamine and that the phosphates were only present in small quantities, if at all.

It seemed possible that the higher values obtained with *Str. faecalis* might be due to the presence of alanine or alanine peptides in the milk extracts. With this possibility in mind we examined the milk extracts by paper chromatographic techniques

which led to the finding that the freeze-dried raw and evaporated milk samples contained appreciable quantities of pyridoxamine phosphate that had not been hydrolysed to free pyridoxamine by the acid treatment used for preparing the milks for assay. In confirmation of our previous findings, fresh milk only contained a small amount of pyridoxamine phosphate. The techniques used to demonstrate the presence of pyridoxamine phosphate in milk extracts, and the effect its presence had on microbiological assays of these samples, will now be described.

EXPERIMENTAL

Vitamin B_6 standard solutions

Stock solutions of pyridoxal hydrochloride (L. Light and Co.) and pyridoxamine dihydrochloride, pyridoxamine phosphate and pyridoxal phosphate (Roche Products Ltd) were prepared at concentrations of 50 μg of free base/ml as described by Gregory (1959).

Milk samples

The freeze-dried raw and evaporated milks were the same as those used in a previous study on the effect of heat on the vitamin B_6 content of milk and their preparation has been described elsewhere (Davies, Gregory & Henry, 1959). The samples were stored in airtight containers in a refrigerator. A sample of bulk fresh milk from the Institute herd was also used. It was stored in the deep-freeze.

Extraction of the vitamin B_6 active compounds from milk

(*a*) *Acid treatment.* Liquid milk (2 ml) or freeze-dried milk (0·5 g) in 90 ml of 0·055 N-HCl was heated in flowing steam for 30 min. After cooling, the volumes were made to 100 ml with water, the pH adjusted to 4·6 and the extracts filtered. The clear filtrates were diluted to appropriate concentrations for assay with the different test micro-organisms as described later.

For chromatographic and electrophoretic examinations of the milk extract, the filtrate was adjusted to pH 2 with 2 N-HCl and passed through a 20 × 3 cm column of Dowex 50 (× 8) H^+ resin. The eluate and subsequent wash water (100 ml) containing sugars and anions was discarded and the adsorbed vitamin B_6 was eluted with 2 N-NH_4OH. Almost all of the active compounds emerged with the NH_4OH front but 30 ml of the NH_4OH eluate was collected to ensure maximal recovery. This active fraction was concentrated by distilling to dryness under reduced pressure at 50 °C and was then redissolved in 20 % aqueous ethanol.

(*b*) *Acid treatment followed by incubation with intestinal phosphatase.* A 20 ml portion of the clear filtrate, prepared by the acid treatment described above, was adjusted to pH 8·0 with NaOH. Two mg of intestinal phosphatase (Armour Pharmaceutical Company), in 4 ml of 0·1 M Na_2CO_3–$NaHCO_3$ buffer at pH 8·5, were added and the extract incubated at 37 °C for 2 h. The volume was then made to 100 ml and the pH readjusted to 6·8. Further appropriate dilutions of the extract were made as necessary for the different test micro-organisms.

(*c*) *Milk ultrafiltrates.* Ultrafiltrates were prepared as described by Gregory (1954) from the liquid milk and from the freeze-dried raw and evaporated milks reconsti-

tuted in water at concentrations of 10 % (w/v). The ultrafiltrates were then treated with four volumes of ice-cold acetone and an inactive precipitate containing mainly lactose was removed by filtration. The acetone was removed under reduced pressure. This acetone treatment enabled the ultrafiltrate to be more easily concentrated on the chromatogram paper by multiple spotting.

Chromatography and electrophoresis

Two-dimensional chromatography was carried out on Whatman No. 1 paper using the solvent systems described by Levy & Chung (1953). Butanol-acetic acid-water (4:1:5) was used as the solvent for one-dimensional development. Paper electrophoresis was done on 30 × 25 cm Whatman No. 1 strips with 0·05 M acetate buffer and an applied potential of 400 V (current 6–7 mA). Initially the buffer was used at pH 5·1 as recommended by Siliprandi, Siliprandi & Lis (1954). Although this method appeared to give good separations of the vitamin B_6 compounds when sufficiently high concentrations were used to allow detection under u.v. light, it was found that when the more sensitive bioautographic method was employed, there was considerable streaking of all components. When milk extracts were examined under these conditions, the streaking made it impossible to interpret the result with certainty. Consequently the pH range of 5·1–8·6 was explored and a pH of 5·8 was found to be optimal.

To detect phosphates of pyridoxal and pyridoxamine, the paper was dried after electrophoresis and sprayed evenly with a 0·1 % (w/v) solution of intestinal phosphatase in 1 % (w/v) aqueous $NaHCO_3$. Sufficient spray was applied to ensure that the pH of the paper was raised to about 8. The moist paper was then incubated in a moist atmosphere for 1 h at 37 °C to release the unphosphorylated forms before the bioautograph was prepared.

Microbiological methods

Tube assays. The methods of assay with *Saccharomyces carlsbergensis* 4228 (ATCC 9080), *Streptococcus faecalis* R (ATCC 8043), *Str. faecium* Ø51 and *Lactobacillus casei* (ATCC 7469) were as described by Gregory (1959).

Bioautograph plates. Agar plates seeded with the different test micro-organisms were prepared for use in conjunction with the paper chromatography and electrophoresis studies. One hundred and fifty ml of the appropriate single-strength basal medium containing 2 % (w/v) agar were used to prepare a 22 × 37 cm plate. For the inoculum, *S. carlsbergensis* was grown for 5 h at 28 °C in the inoculum medium used for the tube assays. The culture was centrifuged and resuspended in 5 ml of sterile 0·9 % (w/v) NaCl solution, 4 ml of this suspension being used to inoculate 150 ml of the sterile agar medium held at 47 °C. The plate was poured immediately after inoculation of the medium. *Str. faecalis* and *Str. faecium* plates were prepared similarly except that the inocula were grown for 24 h at 30 °C. The cultures were then centrifuged, washed and resuspended in 10 ml of 0·9 % (w/v) NaCl solution, 1 ml of the suspensions being used to inoculate 150 ml of medium. At the same time, 100 mg of 2,3,5-triphenyltetrazolium bromide ('Grodex', May and Baker Ltd) dissolved in 4 ml of sterile water, were added to the agar so that the growth zones would show up red on

a colourless background. The dye could not be added to the *S. carlsbergensis* plate, since it inhibited growth of this organism. The chromatogram or electrophoresis paper was placed on the surface of the agar, the plate incubated at 30 °C for 18 h and then examined for zones of growth.

RESULTS

Chromatographic studies

The concentrate prepared from the freeze-dried raw milk by Dowex treatment was first examined in duplicate by one-dimensional paper chromatography. Duplicate spots of a standard amino acid mixture were included on the same paper. Half of the chromatogram was sprayed with ninhydrin and the other identical half placed on an agar plate seeded with *Str. faecalis*. Figure 1 shows that three zones with growth activity for *Str. faecalis* were separated from the milk extract. Zone 2 corresponded to the position of alanine on the ninhydrin-sprayed chromatogram, but there was no growth at the position of alanine in the amino acid mix. Further tests showed that alanine could only be detected by this bioautograph technique if more than 100 μg were present. Calculations from the known alanine content of casein (3·6 % w/w) showed that it was most improbable that this amount of alanine would be present in the milk concentrate.

By testing pyridoxal and pyridoxamine in the same solvent systems, it was shown that growth zone 2 was identical with pyridoxamine and growth zone 3 with pyridoxal. When the bioautograph plate was seeded with *S. carlsbergensis* or *Str. faecium*, growth zone 1 was much less intense. This suggested that it might be the factor in the milk extracts stimulating *Str. faecalis* in tube assays and therefore further attempts were made to identify it.

To separate the active factor present in zone 1 from pyridoxal and pyridoxamine, the milk concentrate ($\equiv$ 1·5 g of milk solids) was applied as a streak 17 in. long to filter paper and developed in one dimension. After the position of the zone on a reference strip was located from a *Str. faecalis* bioautogram, the appropriate area was cut out and eluted with 10 % (w/v) aqueous acetic acid. Chromatography of a portion of the eluate showed that it contained only one active component in the position of zone 1. In addition, however, it was noted that ninhydrin-positive material was also to be found in this position suggesting that a complex of vitamin B_6 and amino acid or peptide might be involved. The remaining eluate was, therefore, dried under reduced pressure and hydrolysed in a sealed tube with 6 N-HCl for 16 h at 105 °C under which conditions pyridoxal and pyridoxamine were known to be relatively stable. Chromatographic examination of the hydrolysate showed that the ninhydrin-positive material had been broken down to form leucine, valine, tyrosine, alanine, threonine, glycine and proline. Also the position of the active zone had changed to one corresponding to pyridoxamine. Since it is known that phosphorylated forms of pyridoxal and pyridoxamine occur naturally, the possibility that the active component of zone 1 was pyridoxamine phosphate had to be considered. To check this possibility the phosphates of pyridoxal and pyridoxamine were examined by one- and two-dimensional chromatography. Two difficulties arose—the acidic nature of the first solvent caused some hydrolysis of pyridoxal phosphate and also all activity was destroyed if the second solvent, phenol, was removed from the paper by oven drying at 60 °C.

However, if the phenol was removed by washing with diethyl ether the loss in activity was minimized. Nevertheless, it was not possible to achieve adequate separation of the two phosphates with these solvent systems. However, the position taken up by the zone 1 component was found to coincide with the pyridoxal phosphate–pyridoxamine phosphate position.

To avoid the possibility of hydrolysis of the phosphates during chromatography, trials were made with butanol-water and butanol-water-ethylamine but although hydrolysis of phosphates was prevented the separation was poor. Eventually electrophoresis was found to be a more satisfactory technique.

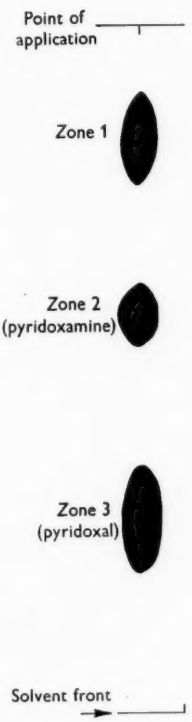

Fig. 1

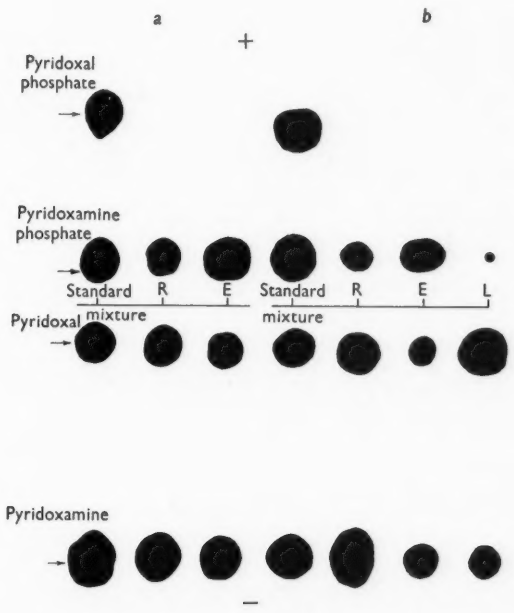

Fig. 2

Fig. 1. Chromatogram of a concentrate prepared from freeze-dried raw milk showing the positions of compounds with growth activity for *Str. faecalis*.

Fig. 2. Electrophoretic separation of the vitamin B_6 active compounds in milk. (*a*) Acid extracts of freeze-dried raw (R) and evaporated (E) milks concentrated by treatment on Dowex 50 resin; (*b*) ultrafiltrates from freeze-dried raw (R) and evaporated (E) milks and from fresh milk (L). The bioautograph plates were seeded with *Str. faecalis*.

Electrophoresis studies

Separation of pyridoxal, pyridoxamine, pyridoxal phosphate and pyridoxamine phosphate was obtained in 0·05 M acetate buffer at pH 5·8 and no hydrolysis of the phosphates occurred. Figure 2 (*a*) shows the positions of the zones of vitamin B_6 activity after electrophoresis of the concentrates prepared from freeze-dried raw and

evaporated milks. With both concentrates there was activity in the pyridoxal, pyridoxamine and pyridoxamine phosphate zones, but no trace of pyridoxal phosphate was detected. There was, however, the possibility that the Dowex treatment of the milk extracts (and in particular the elution with ammonia) had brought about conversion of pyridoxal phosphate and pyridoxal into pyridoxamine phosphate and pyridoxamine. Therefore, the electrophoresis was repeated with milk ultrafiltrates that had been treated with acetone, but otherwise had had no treatment that might bring about changes in the forms of vitamin B_6 present. Fig. 2(*b*) shows that these ultrafiltrates did not contain pyridoxal phosphate. It is also interesting to note from Fig. 2(*b*) that an ultrafiltrate of fresh milk contained only a trace of pyridoxamine phosphate as compared with significant amounts in freeze-dried milk samples. Another interesting feature was that the evaporated milk contained more of the phosphate than the raw milk, a fact which was confirmed by the microbiological tests.

Table 1. *The effect of various treatments of aqueous solutions of pyridoxal phosphate and pyridoxamine phosphate on their activities, assayed by different test organisms, expressed as percentages of the activities of equimolar amounts of pyridoxal or pyridoxamine*

(Mean values are given with the standard error; the number of assays is given in parentheses)

Treatment	*S. carlsbergensis*	*Str. faecalis*	*Str. faecium*	*L. casei*
		Pyridoxal phosphate		
(*a*) None	51 ± 31 (10)	68 ± 12 (7)	70 ± 24 (6)	51 ± 25 (7)
(*b*) Acid	92 (2)	83 (2)	77 (2)	84 (2)
(*c*) Acid + phosphatase	96 ± 8 (6)	103 ± 24 (9)	100 ± 13 (9)	96 ± 24 (8)
		Pyridoxamine phosphate		
(*a*) None	8 ± 12 (10)	113 ± 24 (8)	16 ± 14 (10)	Inactive
(*b*) Acid	34 (2)	101 (3)	29 (2)	Inactive
(*c*) Acid + phosphatase	98 ± 19 (9)	88 ± 15 (11)	90 ± 16 (11)	Inactive

Microbiological studies

Tests with pyridoxal phosphate and pyridoxamine phosphate. A study was made of the effect of acid treatment and acid treatment followed by incubation with intestinal phosphatase on the growth activities of pyridoxal phosphate and pyridoxamine phosphate for the different test organisms. The results (Table 1) showed that untreated pyridoxal phosphate was, in general, less active than pyridoxal for each test organism. The large standard deviations, however, indicate the variability of these results. The reason probably lies in the relative ease with which this phosphate can be hydrolysed. For example, heating at 100 °C for 30 min in dilute acid gave almost full activity for the four test organisms and it seems likely that some hydrolysis took place when the assay tubes containing the test extracts were sterilized at 100 °C for 30 min at pH 6·8. Digestion with phosphatase is seen to be the most reliable method for obtaining full activity with each of the test organisms.

Although pyridoxamine phosphate had only 10–20 % of the growth activity of pyridoxamine for *S. carlsbergensis* and *Str. faecium*, it was fully active for *Str. faecalis*. (Like its free base, it is without growth activity for *L. casei*.) It is less readily hydrolysed by acid than pyridoxal phosphate. Heating at 100 °C for 30 min only increased

the activity to 34 and 29 % of that of pyridoxamine for *S. carlsbergensis* and *Str. faecium*. Again, the phosphatase treatment released full activity for each of the test organisms.

Tests with milk. The freeze-dried raw and evaporated milks and fresh milk were treated with acid and the extracts assayed both without further treatment and after incubation with intestinal phosphatase. The four test organisms, *S. carlsbergensis*, *Str. faecalis*, *Str. faecium* and *L. casei*, were each used to measure the vitamin B_6 activities of the extracts. The mean results of these assays are given in Table 2. To simplify the table only the results calculated from the pyridoxal standards are shown. The increase in vitamin B_6 activity found after phosphatase treatment was assessed by subjecting the results to a two factor analysis of variance, which showed that the treatment did not increase the activity for *L. casei*. With *S. carlsbergensis* there was a 25 % increase in the vitamin B_6 activity of freeze-dried raw milk, 13 % of fresh milk and a very marked increase of 132 % of the freeze-dried evaporated milk. With *Str. faecalis* the increases were smaller but significant in the freeze-dried milks, whereas in the fresh milk the bigger increase of 22 % turned out not to be significant at the 5 % level. There were quite marked increases in activity for *Str. faecium* after phosphatase treatment of both the freeze-dried milks and the fresh milk.

Table 2. *Mean values for the vitamin B_6 activities of extracts of freeze-dried raw and evaporated milk and of fresh milk assayed with the different test organisms before and after phosphatase treatment*

(All results are expressed in terms of pyridoxal (free base); the number of assays is given in parentheses.)

Type of milk	Treatment	*S. carlsbergensis*, μg/g	*Str. faecalis*, μg/g	*Str. faecium*, μg/g	*L. casei* μg/g
Freeze-dried raw	(*a*) acid	2·53 (10)	3·89 (10)	3·31 (8)	2·19 (9)
	(*b*) acid + phosphatase	3·16 (10)	4·51 (10)	4·60 (8)	2·32 (9)
	% *increase*	25**	16*	39***	6 N.S.
Freeze-dried evaporated	(*a*) acid	0·76 (7)	3·59 (6)	1·98 (7)	0·46 (8)
	(*b*) acid + phosphatase	1·76 (7)	3·71 (6)	3·45 (7)	0·52 (8)
	% *increase*	132***	3*	74**	13 N.S.
		μg/ml	μg/ml	μg/ml	μg/ml
Fresh liquid	(*a*) acid	0·47 (4)	0·55 (4)	0·53 (4)	0·45 (4)
	(*b*) acid + phosphatase	0·53 (4)	0·67 (4)	0·74 (4)	0·44 (4)
	% *increase*	13**	22 N.S.	28*	0

Levels of significance: *, $P < 0{\cdot}05$; **, $P < 0{\cdot}01$; ***, $P < 0{\cdot}001$; N.S., not significant at the 5 % level.

From the mean results obtained in these tests, the pyridoxine, pyridoxamine and pyridoxal contents of the milks before and after phosphatase treatment were calculated by the method of Rabinowitz & Snell (1948), except that the *Str. faecium* results were used instead of the *Str. faecalis* results. The calculated values are presented in Table 3. The pyridoxal values were not significantly affected by the phosphatase treatment but the pyridoxamine content was doubled in both the freeze-dried raw and evaporated milks after phosphatase treatment. There was also a four-fold increase in the pyridoxamine content of fresh milk. It is interesting to note

that the evaporated milk contained, on a dry weight basis, more pyridoxamine and pyridoxamine phosphate (calculated from the increase after phosphatase treatments) than the raw milk. Also there was proportionally more pyridoxamine present in the freeze-dried raw milk than in the fresh milk.

Although the calculations indicated that some pyridoxine was present, we have as yet found no direct evidence for the presence of either pyridoxine or pyridoxine phosphate in the extracts of the freeze-dried milks.

Table 3. *Pyridoxine, pyridoxal, and pyridoxamine contents of extracts of freeze-dried raw and evaporated milk and of fresh milk, calculated from values obtained before and after phosphatase treatment*

(Calculated by the method of Rabinowitz & Snell (1948) using results obtained with *S. carlsbergensis*, *Str. faecium* and *L. casei*).

Type of milk	Extraction method	Pyridoxine, μg/g	Pyridoxamine, μg/g	Pyridoxal, μg/g	Total activity, μg/g	Rat† assay value, μg/g
Freeze-dried raw	(*a*) acid	0·09	0·49	2·19	2·77	4·9
	(*b*) acid + phosphatase	0·36	0·99	2·32	3·67	(3·7–6·7)‡
Freeze-dried evaporated	(*a*) acid	0	0·71	0·46	1·17	—
	(*b*) acid + phosphatase	0·55	1·48	0·52	2·55	2·7 (2·0–3·7)‡
		μg/ml	μg/ml	μg/ml	μg/ml	
Fresh liquid	(*a*) acid	0·01	0·03	0·45	0·49	—
	(*b*) acid + phosphatase	0·03	0·14	0·44	0·61	—

† Data from Davies, Gregory & Henry (1959). ‡ Approximate limits of error.

DISCUSSION

In our previous studies of the effect of heat on the vitamin B_6 of milk (Davies *et al.* 1959) we were unable to explain two of our findings. The first of these was that *Str. faecalis* gave higher values, particularly with evaporated milk, than *Str. faecium*, although both organisms were shown to respond about equally to pyridoxal and pyridoxamine. The second unexpected result was that values for the evaporated milk, obtained from growth tests with chicks and rats, were higher than those obtained microbiologically with *S. carlsbergensis*. This micro-organism can utilize pyridoxine, pyridoxamine and pyridoxal about equally for growth and on this account is generally used for measuring total vitamin B_6 activity. The low result which it gave with evaporated milk was at first thought to be due to some factor in the milk extract inhibiting growth. When, in the work now described, it became apparent from the chromatographic and electrophoretic studies that the milk extracts contained pyridoxamine phosphate, then the explanation for the high *Str. faecalis* values and low *S. carlsbergensis* and *Str. faecium* values became clear.

Microbiological tests with pure pyridoxamine phosphate confirmed the findings of Rabinowitz & Snell (1947) that this compound is largely unavailable to *S. carlsbergensis* but is highly active for *Str. faecalis*. It is also active for chicks (Coates & Harrison, 1960) and rats (Henry, 1961, private communication). Rabinowitz & Snell (1947) showed that pyridoxamine was only slowly liberated from pyridoxamine

phosphate by acid hydrolysis. In 0·055 N-HCl a temperature of 125 °C for 3 h was necessary for complete hydrolysis. Under these conditions pyridoxal phosphate was completely hydrolysed in 30 min. If our milk samples contained pyridoxamine phosphate, most of it would not be hydrolysed by the mild acid treatment used in these tests for extracting the vitamin B_6 active compounds (i.e. heating at 100 °C for 30 min in 0·055 N-HCl). Therefore higher values would be expected with *Str. faecalis* as the test organism and this is what we found. Further evidence that the discrepancy in the results given by the different test organisms was due to the presence of pyridoxamine phosphate was obtained by treatment of the acid extracts with intestinal phosphatase. This resulted in an increase in activity measured with *S. carlsbergensis* and *Str. faecium* so that the *S. carlsbergensis* values for total vitamin B_6 activity in evaporated milk then were consistent with those found previously in chick and rat tests (Davies *et al.* 1959). The discrepancy between the *Str. faecalis* and *Str. faecium* results was also decreased.

Although we have established the presence of pyridoxamine phosphate in fresh and freeze-dried milk samples, it is still uncertain whether pyridoxal phosphate is also present. This has proved difficult to determine because pyridoxal phosphate is hydrolysed by the acid conditions of extraction used in our tests. There is also the possibility that it is associated with the milk proteins and for this reason was not detected in the ultrafiltrates.

The reasons for our using the shorter heat treatment at the lower temperature were: (*a*) the effect of heat on the vitamin B_6 of milk was being studied and further prolonged heating was thought inadvisable; and (*b*) preliminary tests with fresh milk and National Dried Milk did not show any increased vitamin B_6 activity after the prolonged heat treatment as compared with the milder treatment. The electrophoretic tests described in this paper indicated only a trace of pyridoxamine phosphate in the fresh milk ultrafiltrate. Further, the microbiological tests showed that there was less pyridoxamine phosphate in the fresh milk than in the freeze-dried raw milk. This explains why in the preliminary tests a mild acid treatment was able to extract most of the vitamin B_6 activity from the fresh milk.

There is the possibility that fresh milk varies in its content of pyridoxamine phosphate. However, our results suggest that some phosphorylation of pyridoxamine had taken place during the processing or storage of the freeze-dried milk samples. The fact that more of the phosphate was present in the freeze-dried evaporated milk lends support to this hypothesis.

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Reviews of the progress of dairy science

Section B. Bacteriology

The flavour of Cheddar cheese

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During the period 1938–59 the total annual production of Cheddar-type cheese in the United Kingdom increased three-fold from 17000 to 50000 tons owing entirely to a higher output of factory made cheese (1). Indeed the annual production of farmhouse Cheddar fell, in the same period, from 4850 to 3150 tons. Production in other large Cheddar-producing areas, the United States of America, Canada, New Zealand and Australia showed a similar trend. The emphasis on mechanization has inevitably involved modifications of the cheesemaking process which have largely been introduced by trial and error methods and this continues apace as novel ideas to facilitate automation of the various stages of manufacture are developed (2, 3, 4). Some of the resulting changes in cheesemaking technique, although superficially innocent, may have profound effects on cheese quality—for example, the use of bulk milk which is likely to have a larger and more varied flora, the use of pasteurization with concomitant chemical and microbiological changes, and the overnight storage of milk either before or after pasteurization which may allow important enzymatic reactions to proceed despite the low temperatures of storage.

The factory product, as would be expected, is usually of more even quality than farmhouse cheese but when compared with the better samples of the latter is, in general, milder and poorer in flavour. A connoisseur might be forgiven for occasionally dreaming of the good old days when Cheddar was as plump as a dairymaid.

The emphasis on factory production has brought an increasing need to know the origin and chemical identity of the cheese flavour components and to understand how they are produced. Such knowledge would enable their concentration in cheese to be controlled either by the addition of suitable synthetic or naturally produced substances or by scientific manipulation of the many variables involved in the making and maturing of cheese. (A good cheese flavour essence would also find ready application in other food products such as biscuits and sauces.)

Investigations during the past fifty years have led to a fairly detailed knowledge of the chemical substances produced during maturation but, apart from the established importance for flavour of the methyl ketones in mould-ripened cheese (5), no major advance in understanding the relationship of composition and flavour has been made.

The subjective nature of flavour assessment has been and continues to be a major obstacle. The interpretation of the most detailed experimental results ultimately depends on a variable physiological and psychological response of a tasting panel, and strict statistical control becomes imperative. Even so the difficulty of describing flavour sensations seriously impedes progress. It would, however, be helpful if workers in this field would clearly distinguish, when possible, between the taste and smell components of flavour.

Several recent reviews have been devoted to cheese flavour with particular emphasis on the chemical aspects of ripening (6,7,8,9). Nevertheless the changes which occur in Cheddar are of chemical and microbiological origin and in this review an attempt is made to consider and relate both aspects in the light of work published in the last decade.

The contribution of bacteria to the development of flavour

The known metabolic products of the different species of homofermentative lactic acid bacteria which occur in cheese—streptococci, lactobacilli and pediococci—are so qualitatively similar that it is difficult, on this basis, to suppose that one species or strain would be of more importance in flavour production than another. However, small differences in the minute amounts of odoriferous or flavourful substances such as diacetyl which are produced could be important. Therefore, the relative numbers of the different species merits attention. It follows too that the numbers of heterofermentative bacteria may be especially significant.

The starter streptococci

It is still not clear what part the starter organisms play in the formation of Cheddar cheese flavour. That the culture used should produce sufficient acid at a convenient rate is a practical necessity of cheesemaking. To this end the selection of suitable strains (10,11), control of phage infection (12,13,14) and other causes of slowness (15), and the stimulation of acid production under adverse conditions continue to be the main subjects of investigation.

The attainment of a correct acidity in the curd at pressing is also a factor controlling subsequent bacteriological, enzymatic and probably chemical changes. In this sense the rate of growth of the starter organisms in cheese-milk has an indirect influence on flavour production. Whether a more direct effect is exerted by the production of metabolites is uncertain. Resting cell suspensions of *Streptococcus lactis* (var. *maltigenes*) in a solution of mixed amino acids have been shown to produce detectable amounts of a range of aldehydes, but much smaller amounts were obtained when a cheesemaking strain of *Str. lactis* was used. *Str. cremoris* produced no aldehyde under the same conditions (16). The inference that some starter organisms may contain oxidative deaminases and decarboxylases which enable them to form odorous compounds from amino acid substrates is useful in relation to other observations that different strains produce different flavours when grown in milk culture (17). Also cheeses made from some strains of *Str. faecalis* have acquired malty flavours derived from the starter metabolism but this is perhaps an abnormal case (18). These observations suggest that different species or strains of starter bacteria may be better than others for the production of Cheddar flavour and might explain the preference of some cheese-makers for commercial cultures (containing mixtures of *Str. lactis*, *Str. cremoris* and leuconostoc spp.). However, the recent demand for a rapid Cheddar cheese process has led to the use of higher temperatures for scalding the curd and has encouraged the use of the heat-tolerant enterococci, *Str. faecalis* or *Str. durans*, as starters (2). Good Cheddar cheese can be made with these bacteria provided the variables of the making process are manipulated to ensure a correct acidity in the green cheese. It follows that even changing the species of starter will have a minimal

effect on flavour provided the acid development is controlled. Nevertheless, some strains or combinations of species have been found to produce improvements in cheese flavour (19, 20).

Perhaps the main contribution of starter to flavour production in cheese is indirect. Since the population of streptococci reaches a maximum of about 10^9/g of cheese in the press considerable enzymatic activity might be expected as the cell enzymes are released by autolysis. Attention has naturally been focused on the proteolytic enzymes of the cell (21). This has been encouraged by the observation that peptide bitterness in cheese occurs if the starter has a limited proteolytic ability which allows the bitter primary breakdown products from cheese protein to accumulate (20, 22, 23, 24). In this respect the death rate of the starter in cheese would be important in determining enzyme concentrations. This rate is determined not only by the environment (salt concentration, acidity, etc.) but also by the strain and species of streptococcus concerned. Thus *Str. cremoris* in cheese has been found to decrease in numbers much more rapidly than *Str. lactis* or *Str. diacetilactis* (25). This observation may be significant in another way, for a rapid decline and autolysis of starter implies a more rapidly changing environment and hence a quicker establishment of succeeding flora.

The lactobacilli

Investigations to ascertain the contribution of the bacteria which develop during ripening to the production of flavour in cheese often seek to correlate the numbers of various species with the quality or intensity of flavour. Such a correlation does not, however, necessarily imply cause and effect for although it is possible that the environment in cheese is determined by microbial development it is equally possible that a chemically produced environment controls the microflora. However, because the lactobacilli reach their maximum numbers and form the major part of the bacterial population in Cheddar cheese at about the time that typical flavour becomes obvious they have for many years been thought to play an important role in flavour development. This view is especially founded on the well-known work of Sherwood (27, 28) but subsequently no good evidence has accumulated to establish the hypothesis.

Recent developments of selective growth media and serological methods of identification have enabled the growth rate and identity of these bacteria to be more carefully studied (29, 30, 31, 32). Their numbers in raw milk have been found to vary widely from about 1 to 10^6/ml but the factors determining their concentration are not yet established. Since their heat tolerance varies with strain and since the conditions of pasteurization vary from factory to factory it is not surprising that in practice the numbers found in the milk after heat treatment are variably decreased (37, 34). In an investigation involving seventeen creameries the majority of milks after pasteurizing contained less than 10 lactobacilli/ml. The decrease in numbers is partially offset by an increase which occurs during cheesemaking due to contact with unsterile equipment or the dairy environment, but the significance of this local flora is not known (35, 36, 38).

The use of selective media for counting the lactobacilli has shown that in cheese they grow from the beginning of cheese making. Mostly a slow growth rate has been reported resulting in maximum numbers of about 10^7/g in 2–4 months (31, 32). This slow growth is explained by the restricted supply of carbohydrate, by the slow release

of easily utilized energy sources and by other inhibitory properties of the environment in cheese (39, 40, 41). However, the rate of growth in some Canadian cheese has apparently been found to be much higher and maximum numbers of 10^6–10^8/g were reached in the first 2 weeks (42). If the lactobacilli contribute to flavour it might be expected that such cheese would acquire mature flavour after a relatively short ripening period. No such observation was reported.

Recent evidence that lactobacilli are important in flavour production has been mainly derived from experiments in which the bacteria were inoculated into the cheese milk and the cheese was compared in flavour with uninoculated controls. Slight or no improvement in flavour was obtained when lactobacilli were inoculated into raw milk cheese (43, 44) but inoculation of pasteurized milk (with lactobacilli from good Cheddar) enhanced the flavour of the resultant cheese (42). It could be supposed that the beneficial inoculum operated by restoring the numbers of lactobacilli in the pasteurized milk to about or above the level present before heat treatment, but there is some evidence that the quality of pasteurized milk cheese is not correlated with the numbers of lactobacilli in the cheese milk (34). Also the final numbers of lactobacilli in the cheese are apparently not necessarily determined by the number present in the milk (43). An attractive hypothesis, therefore, is that the function of the inoculum is rather to supply desirable species or strains which on account of their heat lability are removed during pasteurizing. However, detailed serological studies of a limited number of raw milk cheeses did not indicate that certain species or strains of lactobacilli, including the heterofermentative species *L. brevis*, were more important in flavour production than others (43).

The role of lactobacilli in Cheddar cheese is therefore still obscure. Clarification may be achieved by further inoculation experiments but it is essential that the inoculated organisms should be followed throughout the cheese ripening to establish that growth of the inoculum does occur, and that control cheeses should be checked to exclude the possibility of cross inoculation. Also since the streptococci are known to produce inhibitory and stimulatory compounds (46, 47) and since their death rate may determine the availability of suitable energy sources it is possible that a study of the associative growth of streptococci and lactobacilli would be rewarding. Some indication that the rate of growth of the lactobacilli in Cheddar cheese is higher when the starter flora declines rapidly can already be found (48).

The pediococci

Few investigations of the occurrence of these bacteria in Cheddar cheese have been made. However, data are now accumulating which suggest that they are worthy of more attention. In particular it has been shown that inoculation of cheese milk with pediococci may enhance the typical flavour of New Zealand cheese (45). The organism is incorrectly classified as leuconostoc sp. in this work (50). Subsequently these bacteria have been more frequently isolated from cheese by other workers (33, 49). In the study of their growth in New Zealand Cheddar (48) which was admittedly matured under rather abnormal conditions (waxed 350 g pieces) it was found that in general they were about one quarter as numerous as the lactobacilli throughout the ripening period, but at some stage they became for a short time the dominant genus. An important conclusion was that flavour development in the cheese was more highly

correlated with growth of the pediococci than with that of the lactobacilli but no details of flavour assessment are presented and confirmation of this result is needed.

The micrococci

Despite the well-known proteolytic and lipolytic potential of this group of bacteria and the fact that they have long been known to occur in Cheddar cheese (51) surprisingly little attention has been given to their possible importance in flavour production. One reason for this has been the absence of reports indicating their growth in the cheese during ripening. Also investigations have been hampered by the difficulties of classifying these organisms and of estimating their numbers in cheese in the presence of the dominating lactic flora. The use of a counting medium poor in nutrients has now provided some selectivity and its use has shown that in raw milk cheese multiplication of micrococci may occur, not in the ripening period, but during cheesemaking and curd pressing (52). A thousand-fold increase in numbers to a maximum of 10^{7-8}/g was observed in the 2 days of pressing and was followed by a slow decline over a 3-month period. Since the genus contains members with a high tolerance to heat it is perhaps noteworthy that no similar multiplication could be observed in pasteurized milk cheese despite the presence of micrococci which had survived heat treatment. This fact together with the further observation that inoculation of pasteurized milk with selected cultures from raw milk cheese hastened the development of cheese flavour (53) again suggests that it is the heat-sensitive bacteria which are important. However, the effectiveness of inoculation was based on experiments in which the cheeses were minced up and re-pressed at the innocent age of 2 weeks—a most unfortunate technique. Also it is not stated whether inoculation resulted in any qualitative change in flavour.

In the writer's laboratory the selectivity of the above medium was insufficient to allow the micrococci to be satisfactorily investigated but no better medium could be devised. A recent technique in which the dominating starter bacteria can be eliminated by incorporating bacteriophage in the counting medium provides an improved if restricted method of studying the non-lactic flora (54, 55). Its use has failed to confirm that appreciable growth of micrococci occurs in the cheese at any period. A tenfold increase which was recorded at pressing could be mainly accounted for by the concentration of organisms which is known to occur in the curd during cheesemaking (56). Nevertheless, it is claimed that the inoculation resulted in improved flavour of the cheese. Investigations of food poisoning outbreaks have indicated that *Staphylococcus aureus* in raw cheese milk can multiply during cheesemaking particularly if they are initially present in high numbers (57), and consequently can effectively compete with other bacteria (58). Thus the low numbers of micrococci which are normally present in pasteurized cheese milk may therefore explain their failure to multiply appreciably in the cheese.

If it can be shown by examination of a number of commercially produced cheese that micrococci do not multiply appreciably if pasteurized milk is used for cheesemaking an interesting and potentially important difference between the bacteriology of raw and pasteurized milk cheese will be established.

Other bacteria

It has recently become clear that bacteria which are present in relatively small numbers may nevertheless be of importance in cheese ripening. Indeed it has been shown that bacteria belonging to the genera *Flavobacterium* and *Pseudomonas*, which may multiply in the milk during storage but which do not grow in the cheese, can produce lipolysis of the cheese fat (59). Further this lipolysis may still occur, though on a reduced scale, even if the cheese milk is pasteurized for although the bacteria are destroyed by the heat treatment their lipolytic enzymes are sufficiently heat stable to retain some activity (60). These reports suggest that a good deal more attention should be paid to the microflora of the milk used for cheesemaking. For example it is inferred that milk of relatively poor bacteriological quality may be potentially better for pasteurized cheesemaking.

Similar thinking has instigated work on the effect on flavour of introducing coliform bacteria into the pasteurized cheese milk (62). These micro-organisms are usually associated with body or flavour defects in cheese (63), but it is possible that when present in small numbers their metabolism will result in a fuller cheese flavour. This possibility warrants further investigation. In addition interesting inhibitory or stimulatory relationships between these bacteria and the other components of the microflora of cheese have already been observed (64, 65).

The enterococci (*Str. faecalis*, *Str. durans*) have also been considered as possibly important in cheese flavour production. These bacteria which can be found in raw milk and which have a high heat resistance would be expected, on account of their tolerance of salt and acid, to survive in Cheddar cheese and to be of possible importance because of the proteolytic ability of some of the strains. In raw milk cheese a few millions/g have been found during the ripening period (66*b*). The recent demand for a short time Cheddar cheese process has involved the use of higher scald temperatures and encouraged the use of these enterococci as starters in place of the usual *Str. lactis* or *Str. cremoris*. Although this may have the advantage that flavour development in pasteurized milk cheese is speeded up (66*a*, *b*, 68*a*, *b*) the improvement in flavour, if any, is small enough to indicate that the low numbers found in 'normal' cheese are unlikely to be a major factor in flavour production.

Other bacteria found in Cheddar cheese have been associated with defects but nevertheless it is conceivable that when present in normal concentrations they may favourably influence cheese flavour. For example the leuconostocs (betacocci) which have been frequently found in commercial starters have been shown to be responsible for 'slit-openness' in Cheddar resulting from the large amount of carbon dioxide which they produce (69, 70). The fact that they are heterofermentative suggests that they may be normally of some importance in flavour production.

Recent techniques

As pointed out earlier bacteriological workers have frequently attempted to find a correlation between the composition of the microflora of cheese and the time of appearance of flavour or alternatively have attempted to demonstrate that inoculation of cheese milk with a certain culture will favourably influence flavour production in the cheese. There are, however, so many uncontrollable variables in cheesemaking

that the results of such experiments can be easily misinterpreted. The doubt which still exists (in the writer's mind) as to the importance of micro-organisms in cheese flavour production can be attributed to such difficulties. Of these variables the most difficult to control are the adventitious bacteria which get into the cheese either as components of the cheese milk flora or as contaminants during cheesemaking. If it were possible to exclude these micro-organisms then cheese could be made containing no bacteria or starter bacteria only and the importance of the microflora which develop either during cheesemaking or ripening could be unequivocably assessed. A step in this direction has been made by the successful use of apparatus designed to exclude bacteria from the vat during cheesemaking (71). Its exploitation will depend on the success of methods aimed at controlling the flora of milk. Heat treatment has been used satisfactorily to eliminate lactobacilli from the milk (71) but other methods or combinations of methods such as peroxide treatment (72,73), addition of antibiotics (74), irradiation (75) and high-speed centrifugation (76) will be required to deal with the other bacteria.

These techniques should not only allow the overall importance of bacteria in cheese ripening to be assessed but should later enable the results of inoculation experiments to be safely interpreted.

Biochemical aspects of flavour production

Milk—the substrate

Considering that milk contains all the chemical substances from which cheese flavour is ultimately derived it is astonishing that so little information is available concerning the effect of changes in milk composition on the formation of Cheddar flavour. This is the more surprising in view of the large variations which are known to occur when controllable variables such as breed, nutrition, environment, and stage of lactation of the cow, are altered (9).

Possible changes in the chemical composition of milk fat and protein will be mentioned later but, taking a general example, the ratio of fat to protein in the milk determines the physical structure and moisture-holding capacity of the cheese curd and so controls the subsequent bacteriological and enzymatic changes. In addition the concentration of the minor components of milk may limit the production of small quantities of substances essential for balanced flavour. In this category may be listed citrate (77) for the production of diacetyl, trace metals and other inhibitory or stimulatory compounds for bacteriological growth, and the enzymes of milk (9). The latter, for instance the lipases, proteases and phosphatases, may be of more importance in the ripening of cheese than was formerly supposed and are sufficiently heat stable to retain some activity after the relatively mild pasteurization treatments to which cheese milk is usually subjected (78).

Many investigators have found that the rate of flavour development in pasteurized milk Cheddar cheese is slower and the final flavour is milder than in raw milk cheese (e.g. 79). This effect has usually been attributed to the decrease in bacteria and inactivation of milk enzymes which result from the heat treatment. However, the possibility of important chemical changes arising from pasteurization cannot be ignored, for although no alteration in the electrophoretic pattern of milk protein

occurs under the mild pasteurization conditions used for cheese milk (80,81) there is, for example, a sufficient chemical and/or physical change to decrease the rate of clotting.

Fermentations in Cheddar cheese

Although lactose is by far the most plentiful carbohydrate in milk (4–5 % w/v) it is now clear that other kinds of both free and bound sugars are also present in small amounts. Glucose has been estimated to occur in the free state in concentrations of about 0·006 % and galactose, mannose, lactose and glucosamine combined with milk protein to contribute at least a further 0·05 % (82,83,84). Since most of the whey protein is lost during cheesemaking the bound sugar in green cheese is mainly that associated with casein, namely galactose with small amounts of mannose (84). In addition small amounts of these and the other sugars are bound to the whey protein in the curd. Because of the concentration of casein involved during cheesemaking the bound galactose content of the curd will be of the order of 0·1–0·8 %. It is also possible that the fermentation of lactose by the starter streptococci may be accompanied by the production of traces of polygalactose (85), but of more importance is the polysaccharide incorporated into the streptococcal cell walls. This is built up of glucose, galactose, mannose, rhamnose and glucosamine and since these organisms reach numbers of over 10^9/g of cheese it can be calculated from available data (86) that their sugar content would contribute a further 0·01 % to the cheese.

These bound forms of sugar will be unavailable for bacterial growth until released by breakdown of the milk protein or bacterial cell walls and dissolution in the aqueous phase.

The utilization by the starter bacteria of the lactose in the cheese milk and curd is very rapid and it has been reported that lactose and its component sugars, glucose and galactose cannot be found in Cheddar cheese after a few days from pressing (87). Bound forms of sugar would, however, be expected to be released during ripening and the small amounts expected (0·03–0·1 % w/w) have been observed (39) and consist mainly of lactose and galactose. If these sugars are utilized by the cheese microflora the maintenance of sugar concentration will be dependent on the rate of their liberation from protein and this process, therefore, may control the growth of the lactobacilli and the other bacteria which develop during ripening.

Almost all of the lactic acid bacteria which multiply in Cheddar cheese are homofermentative but this description is relative. Thus some strains of starter streptococci can produce small but significant amounts of diacetyl and acetic acid from sugar (88,89) which contribute to the flavour of new curd. Fermentation of sugar by heterofermentative bacteria would be expected to result in a greater quantity and range of flavourful products but this is only possible in Cheddar cheese in the later part of the ripening period when the appropriate bacteria e.g. *L. brevis*, *Leuconostoc* spp. have developed. At this time, however, sugar is available in only minimal quantities and consequently the products of heterofermentation, e.g. acetic acid, ethanol, glycerol and mannitol (90) will be released in only small amounts. However, the alcohol produced may be sufficient to allow the subsequent chemical formation of significant amounts of ester. Appreciable amounts of formic acid have also been detected in Cheddar type cheese. The origin of this is not certain. It could arise as a result of

fermentation by the coliform bacteria but there is also a suggestion that it could be a product of fat hydrolysis (91).

The fermentable substrate present in Cheddar cheese in the highest concentration during ripening is of course lactate which in Swiss cheese is converted by propionibacteria into propionates of characteristic flavour. However, in normal Cheddar cheese there is no evidence that any bacteria present can utilize lactate as energy source or that propionic acid is normally present.

Citrate in cheese may be another important source of flavour compounds. From this substrate and under certain conditions species of the genus *Leuconostoc*, strains of *Str. lactis* (*diacetilactis*) and *L. brevis* which have been found in Cheddar cheese are capable of producing diacetyl, and possibly acetylmethylcarbinol, 2,3-butylene glycol, acetic and propionic acids with traces of alcohols and aldehydes of which diacetyl contributes most to the aroma (90). The citrate content of milk is, however, small, 0·1–0·18 % (92*a*) and varies especially with season or stage of lactation (92*b*) being highest in bulk milk samples during May–July. Since it is in this period that milk quality is optimum for Cheddar cheese making it would be worth while if citrate fermentation in Cheddar cheese were more closely examined. This view is reinforced by the now old report that the diacetyl content of cheese falls during ripening as a result of some process which is not understood and that relatively low values of diacetyl ($< 0{\cdot}05$ mg/100 g) are found in good quality cheese (26).

Proteolysis in cheese

The pathway of proteolysis in cheese has now been somewhat clarified by the use of electrophoresis to characterize the proteins and peptide fractions and of chromatography to identify and measure the smaller peptides and amino acids. There is agreement that the electrophoretic pattern of the cheese protein is dependent on the variety of the cheese and on its state of maturity (93,94). If mature cheeses are compared the pattern is in general characteristic of the cheese variety. The casein fractions in cheese appear to be attacked with shifting emphasis in a sequence which is common to most varieties and the final result is largely determined by the stage of proteolysis reached at the time of sampling or before enzyme activity ceased. The common sequence of changes which involves in particular a component of α-casein, β-casein, and α-casein itself, suggests that rennin is mainly responsible for these early phases of protein breakdown (93). However, the contribution of bacterial proteases to this process cannot yet be assessed. Growing cultures of lactic acid bacteria are capable of utilizing caseinate (95,96) and autolysis of bacterial cells results in the liberation of proteolytic enzymes. However, the proteases of bacteria from cheese which have so far been examined have an optimum pH of 6 or higher and do not therefore appear to be very active in Cheddar cheese (97,98). The role of milk protease, whose existence and tolerance of pasteurization now seems to be established, is equally doubtful (99,100).

Examination of the alcohol-soluble nitrogen fractions in cheese has revealed a large number of components of different sizes and types whose distribution pattern is also characteristic of the cheese variety and presumably reflects varietal differences in the intermediate stages of protein breakdown (101). Whether these differences are due to the activity of specific bacterial peptidases or whether they arise because these

enzymes are presented with different polypeptide substrates depending on the extent and range of the earlier proteolytic processes is not known.

A further complication is that the contribution of rennin and other enzymes present as impurities in the commercial rennet preparations used in cheesemaking to the formation of these smaller peptides and further to amino acids is still uncertain (102). These aspects are clearly pertinent to the flavour problem in view of the connexion between peptides and bitterness (p. 305). This has been further emphasized by the report that bitterness in cheese can result if high concentrations of rennet are employed (103). If this is considered together with the hypothesis that peptide bitterness occurs when the starter bacteria are insufficiently proteolytic it may be concluded that a proper balance is necessary between the concentration and activity of rennet and the bacterial enzymes (104).

The spate of papers dealing with amino acids in cheese is now diminishing as the importance of these compounds in cheese flavour formation becomes better understood. This is particularly true of Cheddar cheese in which their direct contribution to flavour is now clear. The earlier suggestions that specific flavours in cheese may be due to the presence of certain amino acids have in general not been confirmed. That they make an important contribution to the flavour of Cheddar cheese was suggested by early observations that their concentration in raw milk cheese with full flavour greatly exceeded that in the milder pasteurized product (e.g. (105)). It was further shown that the amino acids present in the mature cheese were, not unexpectedly, qualitatively similar to those found in casein (106). This is indeed true of all the cheese types examined. Some interesting differences however are found. For example, in Cheddar cheese ornithine occurs and is doubtless synthesized by the microflora (107). Quantitatively, the pattern of amino acids differs from that of casein and reflects the fact that different amino acids are liberated at different rates (108) and some may be further metabolized.

When amino acids were incorporated into fresh cheese curd in concentrations suggested by the quantitative analyses the mixture was reported to have no cheese flavour (108). It is presumed that this should have been reported as no *Cheddar* (i.e. typical) cheese flavour for it is now obvious that such a blend of amino acids as used would have a brothy taste. This has indeed been observed and confirmed by tasting the peptide-amino acid fraction of Cheddar cheese isolated by ion exchange technique (107), or indeed by tasting Cheddar cheese, made from skim-milk, in which the brothy flavour predominates (39). It is apparent, therefore, that the amino acids only directly contribute a broth-like flavour to Cheddar cheese which is not the typical flavour but which forms an important background on which the typical flavour is superimposed and by which it is enhanced—a hypothesis earlier postulated by Mulder (109).

The further degradation of amino acids in cheese introduces further interesting possibilities. Recent work has shown a close correlation between the concentration of H_2S and Cheddar flavour (110) and suggested that the gas is a product of the metabolism of lactobacilli. It would seem that H_2S makes a useful contribution to the typical cheese aroma (111). Presumably it is formed by the breakdown of the amino acids cysteine, cystine, cysteic acid (107) or methionine and although there is no good evidence that these decrease in concentration during ripening the flavour threshold of

sulphides, especially organic sulphides, is extremely low (a few parts/2×10^{10}) so that a very small conversion of amino acid would be involved. Nevertheless, it would seem that the metabolism of sulphur compounds in cheese is worth careful study and since the production of H_2S may be only an indicator of flavour production the simultaneous formation of other compounds, e.g. methyl sulphides (112,113) merits special attention. The enzymatic approach of Schormüller could be usefully employed in this direction (e.g. (114)).

Methionine has also been involved in another interesting hypothesis of cheese flavour formation which suggests that a slow chemical interaction, over the long period of ripening, between amino acids and dicarboxyl compounds (Strecker degradation), could lead to the production of flavourful aldehydes containing one carbon atom less than the original amino acid (115). A trial of various possible substances indicated that methional had a cheese-like odour. This compound, for example, could be derived as follows:

$$\underset{\text{methionine}}{CH_3\,S\,CH_2\,CH_2\,\underset{\displaystyle NH_2}{\underset{\cdot}{CH}}\,COOH} + \underset{\text{pyruvic acid}}{CH_3\,CO\,COOH} \longrightarrow \underset{\text{methional}}{CH_3\,S\,CH_2\,CH_2\,CHO} + \underset{\displaystyle NH_2\ \text{alanine}}{\underset{\cdot}{CH_3\,CH}\,COOH} + CO_2$$

However, the cheesiness of methional odour has not only been disputed but claimed to be negligible and the previous observations attributed to oxidised impurities (116). In reply the possible importance of methional in cheese flavour has been vigorously re-asserted (117) and its presence in Cheddar cheese demonstrated (118). Since this compound also appears to be responsible for the sunlight off-flavour of milk (9) and possibly important in the flavour of cooked meat (119) it would seem to have rather versatile properties. A further report describes its odour as of raw pumpkin when concentrated but like toasted cheese when dilute (120). In addition 3-methylbutanal, which has a malty aroma and which could be derived from leucine by Strecker degradation, has also been found in Cheddar and thought to contribute flavour (121). It should be noted that these aldehydes are the ones previously referred to (p. 304) which can be produced from amino acids by certain strains of *Str. lactis* as a result of oxidative deamination (16,122). Thus it is still not certain by which mechanism they are formed in cheese.

Lipolysis in Cheddar cheese

This subject which has been dealt with in a recent review by Sjøstrom (123) has in the last decade acquired a new significance in connexion with typical flavour as a result of a number of observations which are suggestive rather than conclusive. It has been observed for instance that no typical flavour develops in 'Cheddar' cheese made from skim-milk (39). This suggests that milk fat is the substrate for typical cheese flavour formation but many other explanations could be invoked. In addition, however, the typical flavour has been found to be soluble in fat solvents and to be concentrated in the fat phase of cheese (124,39). It has also been found that increasing the lipolysis in the cheese by the addition of enzymes or by homogenization can improve the flavour

(125) but excessive lipolysis causes bitterness or rancidity. Of especial interest is the improvement obtained recently in the flavour of pasteurized milk cheese by inoculation of the cheese milk with *Geotrichum candidum* 18 h before cheesemaking, the improvement being accompanied by an increase in concentration of C_6 and higher acids (126). The inference that these acids are in some way important in typical Cheddar flavour production has been further supported by other investigators (127), and these observations may not be unconnected with the fact that ageing cream results in an increase in water-insoluble fatty acids and at the same time produces a cheesy flavour (128, 129). These pointers are the more stimulating because so little is known fundamentally about the lipolytic processes in cheese. Investigations of the activity and properties of the lipase systems in milk and cheese are urgently required and have been facilitated by the advent of gas chromatographic methods for the analysis of fatty acids (e.g. (130)).

Lately the views on the importance of milk lipase in Cheddar cheese ripening have been re-orientated. This has resulted from the demonstration that milk contains not only lipase active at alkaline pH, i.e. inactive in Cheddar cheese, but also around pH 5 and 6 (78). It is, however, clear that much of the lipolytic activity is destroyed by pasteurization even under those conditions which are used for cheese milk (126, 131) but since the lipases are intimately associated with the milk casein there is a concentrating process during cheesemaking. Also it is likely that enzymes with different pH optima vary in their resistance to heat. It would be of great interest if the acid lipase was found, like the acid phosphatase (9) to have a high heat stability.

The high heat tolerance of bacterial lipases and the consequent possible importance of the flora of milk before pasteurization has also stimulated new lines of thought (see p. 308). It has also to be remembered that even non-lipolytic organisms, e.g. lactobacilli, have been shown to have lipolytic properties after autolysis (132, 133). In cheese, therefore, there must be a number of lipolytic systems having different pH optima and also different specificity with regard to substrate and ester bond (134). Whether lipolysis in Cheddar cheese is directed predominantly against certain fat fractions or against certain types of bond still remains to be determined. It requires a careful comparison between the composition of the free fatty acids in cheese and the composition of the acids combined in the various fat fractions.

It follows that if lipolysis is important in cheese flavour formation then so probably is the composition of the milk fat. Since this can be varied by altering the feed, plane of nutrition and environment and since it is also dependent on the stage of lactation of the cow (135) investigation of the effect of fat composition on Cheddar flavour is now desirable. Such studies might well explain for example why the quality of Cheddar cheese is highest when summer milk is used.

The further breakdown of fatty acid to methyl ketones by a β oxidation process is known to occur in mould-ripened cheese and to be responsible for their characteristic flavour. Keto compounds in Cheddar cheese, derived from carbohydrate, amino acid or fat, have been the object of intense study in the last few years and for convenience are considered together below.

Carbonyl compounds in Cheddar cheese

Failure to attribute Cheddar flavour to any of the major constituents of ripe cheese and the conclusion of Dacre (124) that the components of typical flavour are volatile and are present in Cheddar in concentrations of only a few ppm. has, in the last 5 years, led investigators to examine more closely the minor volatile components of cheese and in particular the carbonyl compounds. The results obtained in the more important contributions in this field are summarized in Fig. 1. The qualitative agreement among the different workers is very impressive. It seems certain that the ketones which contain odd numbers of carbon atoms are derived by β oxidation of the

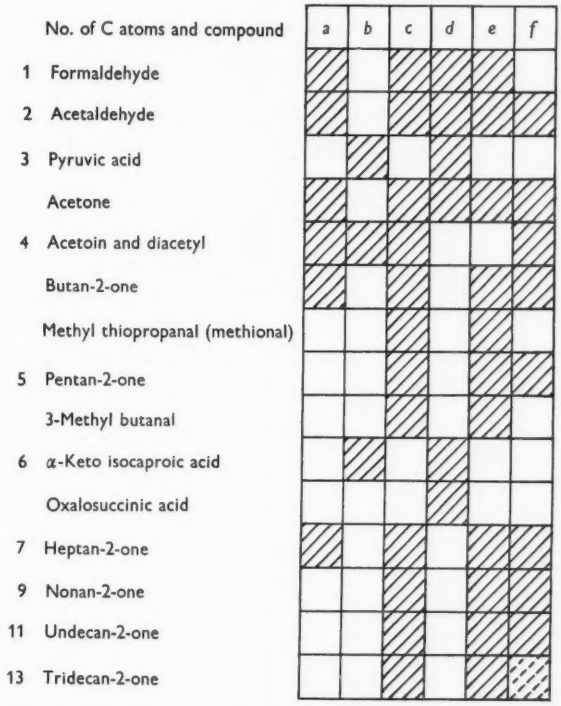

Fig. 1. Carbonyl compounds found in Cheddar cheese by various investigators. *a*: Patton, S., Wong, N. P. & Forss, D. A. (1958). *J. Dairy Sci.* **41**, 857. *b*: Bassett, E. W. & Harper, W. J. (1958). *J. Dairy Sci.* **41**, 1206. *c*: Day, E. A. & Keeney, M. (1958). *J. Dairy Sci.* **41**, 718. *d*: Kristofferson, T. & Gould, I. A. (1959). *Int. Dairy Congr.* **2**, 720. *e*: Day, E. A., Bassette, R. & Keeney, M. (1960). *J. Dairy Sci.* **43**, 463. *f*: Walker, J. R. L. & Harvey, R. J. (1959). *J. Dairy Res.* **26**, 265.

C_4—C_{14} fatty acids of milk fat and this view is supported by the detection of a number of appropriate keto acids (136). Examination of the flavour of suitable mixtures of ketones when incorporated into cheese curd together with a suitable mixture of fatty acids showed that in one case a typical cheese flavour was not produced. However, the quantitative aspect of the analyses is unsatisfactory and other tasting

trials in which the concentration of ketones has been varied and supplemented with a fatty acid mixture together with H_2S have given more promising results (141). It would indeed appear that, if this latest result can be substantiated, the solution of the flavour problem in Cheddar cheese from the synthetic aspect may now come from carefully controlled tasting trials rather than by way of new chemical information. It is expected that emphasis will soon move towards investigation of the conditions (microbial or chemical) which determine the concentration of carbonyl compounds in Cheddar.

Finally it should be noted that all the evidence now suggests that the typical flavour of cheese is due to a complex mixture of components and the success of tasting trials will depend on the care given to obtain the correct 'component balance' (142).

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